DOCTOR OF PHILOSOPHY

Investigating the response of the NEDD8 ubiquitin-like molecule to diverse stress conditions

Leidecker, Orsolya

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By

Orsolya Leidecker

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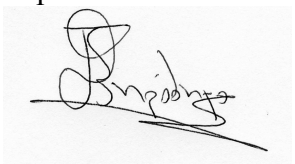
Declarations

I hereby declare that the following thesis is based on the results of work conducted by myself, and that the thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the relevant researchers or their publications. The experiments presented here have, to my knowledge, never been presented for a higher degree or publication excluding where clearly stated and referenced. A large part of my thesis is published in a manuscript accepted for publication in Cell Cycle. The figures in the manuscript and their description are included in this thesis.

Orsolya Leidecker

I certify that Orsolya Leidecker has done the work of which this thesis is a record, and that she has fulfilled the conditions of Ordinance General No. 39 of the University of Dundee and is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Dr. Dimitris Xirodimas
Supervisor



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Abbreviations

ActD	actinomycin D
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
Apaf 1	apoptotic protease-activating factor 1
APC/C	anaphase-promoting complex/ cyclosome
APPBP1	amyloid beta precursor protein binding protein 1
ATP	adenosine triphosphate
BCA3	breast cancer-associated protein 3
BCL	B Cell Lymphoma
BH domain	Bcl-2 Homology domain
BRCA1	Breast Cancer 1
BSA	Bovine Serum Albumin
CAND1	cullin-associated and neddylation-dissociated 1
Caspase	Cystein Aspartate protease
Cbl	Casitas B-lineage Lymphoma
Ced	cell death abnormality
CEP-1	C. elegans-p53
ChIP	Chromatin Immunoprecipitation
CHO	Chinese hamster ovary
CHX	cycloheximide
CID	Collision-Induced Dissociation
Cif	cycle inhibiting factor
CP	core particle, 20S particle of the 26S proteasome
CRL	Cullin Ring Ligase
CSN	COP9 signalosome
Cul	cullin
DCN1	defective in cullin neddylation 1
DDB1/2	damaged DNA binding protein ½
DEN1	Deneddylase-1
Dox	doxorubicin
DTT	Dithiothreitol
DUB	deubiquitinating enzyme
E6AP	E6-associated protein
EDTA	ethylene diamine tetra acetic acid
EGFR	epidermal growth factor receptor
Egl	EGg Laying defective
ENU	Ethylnitrosourea
ER	Endoplasmic Reticulum
FA	Fanconi anemia
FANCD2	Fanconi anemia complementation group D2
FAT10	F-adjacent transcript-10
FCS	fetal calf serum

GST	glutathione S-transferase
Gy	Gray
HA	hemagglutinin
HCD	High energy CID
HECT	Homologous to E6-AP Carboxy Terminus
HIF1α	hypoxia-inducible factor-1
HPLC	High-performance liquid chromatography
IAP	Inhibitor of APoptosis
IgG	immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	ionizing radiation
ISG15	interferon stimulated gene 15
Lys-C	Lysyl endopeptidase C
mA	milli Ampere
Mdm2	murine double minute (note: in this thesis 'Mdm2' is used for the human homologue as well)
MES	2-(*N*-Morpholino) Ethane Sulfonic acid
MHC	Major histocompatibility complex
min	minute
Ml	milli liter
MOPS	3-(N-morpholino)propanesulfonic acid
MS	mass spectrometry
Mtb	*Mycobacterium tuberculosis*
NAE	NEDD8-activating enzyme
NEDD8	Neural Precursor Cell-Expressed Developmentally Downregulated-8
NEDP1	NEDD8-specific protease 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGM	nematode growth media
NT	non-target
NUB1	NEDD8 utimate buster 1
NUB1L	NEDD8 utimate buster 1 long
OD	Optical Density
OTU	ovarian tumor-related
PARP	Poly(ADP-Ribose) Polymerase
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PCR	polymerase chain reaction
pH	power of hydrogen
PH	pleckstrin-homology
PLIC1-4	protein linking IAP to the cytoskeleton 1-4
PML	promyelocytic leukaemia
PRU	pleckstrin-like receptor for ubiquitin
PTM	posttranslational modification
Pup	prokaryotic ubiquitin-like protein
PVDF	polyvinylidene fluoride
RanGAP1	Ran GTPase-activating protein 1

RING	really interesting new gene
RNF4	Ring Finger protein 4
ROS	reactive oxygene species
RP	regulatory particle, 19S particle of the 26S proteasome
rpm	Rotation Per Minute
RT	Room Temperature
Rub1	related to ubiquitin 1
SCF	Skp1/Cul1/F-box complex
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SENP8	Sentrin-specific protease 8
SILAC	Stable isotope labeling by amino acids in cell culture
SIM	SUMO-interacting motif
SMAC	Small Mitochondria-derived Activator of Caspases
SUMO	small ubiquitin-like modifier
TAP	Tandem affinity purification
TBS	Tris buffered saline
TNF	tumor necrosis factor
ts	temperature sensitive
Ub	ubiquitin
UBA	ubiquitin-associated domain
UBD	ubiquitin-binding domain
Ubl	ubiquitin-like molecule
UBL	ubiquitin-like domain
UBZ	ubiquitin-binding zinc finger
UCH	ubiquitin C-terminal hydrolase
UFD	ubiquitin-fold domain
UIM	ubiquitin interacting motif
Ulp-3	ubiquitin- like protein specific protease-3
UPS	ubiquitin-proteasome system
Urm1	ubiquitin-related modifier 1
USP	ubiquitin-specific protease
UTR	untranslated region
V	Volts
VHL	von Hippel-Lindau
v/v	volume/ volume
w/v	weight/ volume
WT	Wild Type
YE	yeast extract
YES	yeast extract+ supplements

Abstract

NEDD8 modification of proteins is extensively studied in the recent years, and the ubiquitin-like molecule has been shown to be involved in numerous signalling pathways. In addition to its well-established roles, we showed that NEDD8 responds to various stress conditions, such as inhibition of the 26S proteasome, heat shock and oxidative stress.

Modification of proteins with ubiquitin and ubiquitin-like molecules is involved in the regulation of almost every biological process. Historically, each conjugation pathway has its unique set of E1, E2 and E3 enzymes that lead to activation and conjugation of their cognate molecules. We also showed the unexpected finding that the ubiquitin E1 enzyme Ube1 activates the ubiquitin-like molecule NEDD8. The above-mentioned stress conditions cause a global increase in NEDDylation. Surprisingly, this does not depend on the NEDD8 E1 activating enzyme but rather on Ube1. A common event in the tested stress conditions is the depletion of “free” ubiquitin. A decrease in “free” ubiquitin levels in the absence of additional stress is sufficient to stimulate NEDDylation through Ube1. We also performed mass spectrometric analyses to investigate NEDD8 chain formation under stress. We found that NEDD8 forms chains with itself and with ubiquitin, and these chains are recognized by proteasome receptors and shuttle factors. Our studies revealed an unprecedented interplay between NEDD8 and ubiquitin pathways, operating in diverse cellular stress conditions.

In a parallel project, we characterized the role of the deNEDDylating enzyme NEDP1 in response to DNA damage. The NEDP1 ortholog in *C. elegans*, Ulp-3 has been previously investigated in collaboration with Anton Gartner’s group. The enzyme has been found to be required for DNA damage-induced apoptosis in the worm germ line.

Our results in human cell lines showed that the role of NEDP1 is conserved, since NEDP1 knockdown resulted in impaired effector caspase activation. Moreover, we showed that the *nedp1* gene is induced upon ionizing irradiation. In the absence of the enzyme, we observed increased NEDDylation that was dependent on the NEDD8 E1 enzyme.

Chapter 1: Introduction

1.1. The history of ubiquitin

Ubiquitin was first identified in 1975 as a 76 amino acid polypeptide that is expressed in all eukaryotic cells[1]. Shortly after, covalent attachment of ubiquitin to target proteins was found to be a major protein regulatory mechanism.

The ubiquitin field was created in the late 1970s, when the ubiquitin conjugation pathway and its role in protein degradation were discovered. In 2004, Avram Hershko, Aaron Ciechanover and Irwin A. Rose were awarded with the Nobel Prize in Chemistry for these discoveries. In the initial experiment, they noted that proteins added to reticulocyte extract became attached to ubiquitin and subsequently degraded by a protease in an ATP-dependent manner[2, 3]. In a paper in 1980, they speculated for the action of a conjugation machinery, the protease function that degrades proteins and the recycling machinery (deubiquitinating enzymes), which amazingly proved to be correct[4]. Later on, the E1, E2 and E3 enzymes were isolated and characterized[5, 6], and the ATP-dependent protease was shown to be the 26S proteasome[7].

The first biological functions for the ubiquitin system were discovered in Alexander Varshavsky's laboratory between 1984-1990. They showed that ubiquitin conjugation was essential for protein degradation *in vivo*[8], required for cell viability and the regulation of stress responses, cell cycle, protein synthesis and DNA repair[9-11]. The first protein identified to be modified by ubiquitin in cells was histone H2A[12] (the role of histone ubiquitination is further discussed in Chapter 1.3).

These early discoveries were followed by the characterization of the enzymes involved in ubiquitin conjugation and the different polyubiquitin chain topologies that play role in distinct cellular processes. Malfunctions of the ubiquitin system have been shown to be linked to disease and aging, making the pathway an attractive therapeutic target.

Nowadays pharmaceutical companies invest a lot in gaining a mechanistic insight into the system and develop compounds that target components of the pathway. The ubiquitin system is still one of the most dynamic and fastest expanding fields in biological sciences.

1.2. Ubiquitin conjugation system

In mammals, ubiquitin is encoded in 4 genes, either as polyubiquitin precursors or as a protein fused to ribosomal proteins L40 or S27a (Figure 1.1).

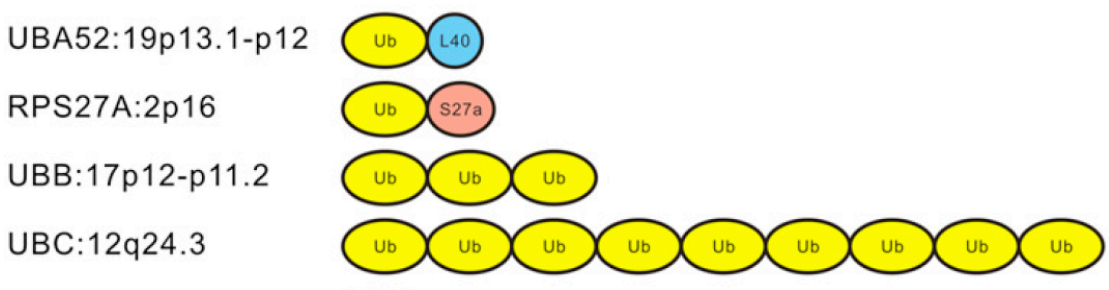


Figure 1.1: Human ubiquitin genes and their chromosomal locations.

Ubiquitin is encoded by 4 genes, and is transcribed and translated either fused to the C-terminus of ribosomal proteins, or as a linear fusion of multiple ubiquitins. Adapted from:[13]

C-terminal hydrolases catalyse cleavage of the fusions, exposing the diglycine motif on ubiquitin that can be activated by one of the two ubiquitin activating enzymes, Ube1 or Uba6[14]. These enzymes share a region of sequence homology with the bacterial enzyme MoeB that catalyse similar reaction. Structural studies on MoeB in complex with MoaD -the small protein in bacteria displaying the ubiquitin-fold and the Gly-Gly motif- provided insight into the mechanism of ubiquitin activation by the E1 enzyme[15].

Ubiquitin E1s consist of three functional parts: an adenylation domain that binds to ubiquitin and ATP, the catalytic cysteine domain and the ubiquitin-fold domain (UFD), which binds to E2 enzymes [15-17]. The E1 enzymes have distinct preferences for E2 charging, and ubiquitin E2s discriminate between Ube1 and Uba6 by the recognition of differences in their UFDs[18].

In the first step of activation, a ubiquitin C-terminal acyl-adenylate formation is catalysed by the E1 enzyme, which binds to ATP-Mg^{2+} and ubiquitin. Next, the C-terminal carboxyl group of ubiquitin forms a thiolester bond with the catalytic cysteine residue in the E1 enzyme. The activated ubiquitin is then transferred to the catalytic cysteine of E2 conjugating enzymes[14]. E2s share a conserved globular domain and some of them also have N- or C-terminal extensions, which may regulate E3 association, intrinsic E2 activity, or substrate recognition[19]. E2s can determine the type of the polyubiquitin chain linkage by orienting the ubiquitin to expose the desired Lys residue to its active site[20]. Ubiquitin is then transferred to the ε-amino group of the substrate lysine via the aid of E3 ligases that contain both E2 and substrate binding sites[21]. One class of the E3s is the HECT domain (homologous to E6-AP carboxy terminus; an approximately 350-amino-acid C-terminal region) containing ligases. Ubiquitin is transferred from the E2s to the catalytic cysteine of the HECT-type E3s; therefore these ligases are directly involved in catalysing substrate ubiquitination[22]. E6-AP, the first E3 ligase discovered in humans belongs to this class of enzymes. It has been shown to target the tumor suppressor p53 for degradation together with the E6 protein encoded by human papillomaviruses[23]. Another predominant class of the E3s is the family of the RING-finger (really interesting new gene)/U-box ligases. RING E3s contain a RING motif (a short motif of $Cys_3HisCys_4$) that coordinates a pair of zinc ions. RING and U-box E3s provide the platform where charged E2s and substrates can bind to close proximity[24]. Recently, two papers have shown that RING/U-box E3s catalyse

ubiquitination by triggering E2-Ub to shift to a more closed, active conformation[25, 26].

Under certain circumstances, E4 ubiquitin ligases can extend ubiquitin chains in conjunction with E3 ligases[27, 28].

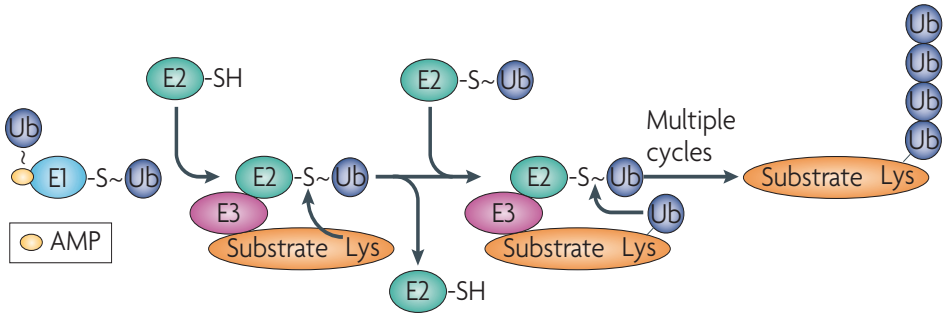


Figure 1.2: The ubiquitin conjugation cascade.

Ubiquitin is activated by the E1 enzyme, then transferred onto the catalytic cysteine of an E2 conjugating enzyme. Through the action of E3 ligases, ubiquitin is covalently attached to substrate lysine residues. Polybiquitin chains are often formed on substrates. Adapted from[14]

Ubiquitination is a reversible process: deubiquitinating enzymes (DUBs) are responsible for cleaving the isopeptide bond between ubiquitin and its target protein. By processing polyubiquitin chains, they have the potential to alter the fate of the substrates (rescue proteins from degradation). On the other hand, once the ubiquitinated protein is recognized by the 26S proteasome, the removal of ubiquitin from substrates by the DUBs is required for protein degradation and recycling of free ubiquitin[29] (Figure 1.3).

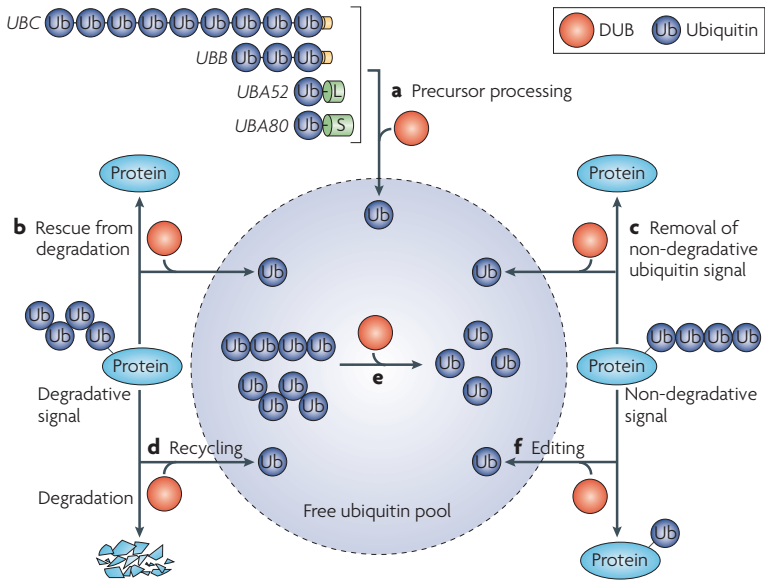


Figure 1.3: The different roles of the deubiquitinating enzymes (DUBs).

(a) DUBs are responsible for the generation of free ubiquitin from the precursors. (b) They also catalyse ubiquitin chain removal from substrates, therefore rescuing them from degradation. Polyubiquitin chain removal prior to substrate degradation by the 26S proteasome, and the trimming of the ubiquitin chains are required for the maintenance of the free ubiquitin pool, and are also catalysed by DUBs (d-f). Adapted from: [29]

The human genome encodes around 100 DUBs. Among them, there are 4 classes of the cysteine proteases: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), Machado-Joseph domain proteases and the ovarian tumor-related (OTU) proteases. They rely on a catalytic diad or triad for their activity (the catalytic Cys, a nearby His and an Asn or Asp residue)[29]. The fifth class of DUBs, the JAMM-motif proteases are zinc metalloproteases. They coordinate two zinc ions that are important for their catalytic mechanism of action[30]. Most of the DUBs catalyse a proteolytic reaction between the ε-amino group of lysine and a carboxyl group on the C-terminus of ubiquitin or ubiquitin-like molecule (ubl).

DUBs possess different types of ubiquitin-binding domains (UBDs) in their structures that mediate protein-protein interactions. The Ile36 patch (Ile36, Leu71 and Leu73) on

ubiquitin has been shown to be important for recognition by DUBs[31]. The enzymes display specificity both to ubiquitin chain types and to substrates and possess a great influence on the outcome of ubiquitin signalling.

1.3. Ubiquitin chain linkages and the biological outcome of ubiquitination

Ubiquitination on proteins can serve as signal for diverse cellular processes. Depending on whether it is conjugated as a monomer or chain of different linkages, it can target substrates to distinct fates.

The first protein identified to be modified by ubiquitin in cells is histone H2A[12]. A single ubiquitin attachment to H2A or H2B has been shown to play critical roles in transcription regulation and DNA repair (reviewed in[32]). Monoubiquitination or multiple monoubiquitination on proteins can also signal for destruction by the lysosomes. Several ion channels and signal-transducing receptors are monoubiquitinated in response to an extracellular signal, and ubiquitination regulates their endocytic transport[33, 34]. Modification by a single ubiquitin also has a crucial role in maintaining genomic integrity, since monoubiqitination of FANCD2 is essential for the Fanconi anemia tumor suppressor pathway to function[35]. Additionally, single ubiquitin attachment has been reported to be important for virus budding[36].

Ubiquitin contains seven lysines (which are Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and all of them can serve in the formation of polyubiquitin chains. Ubiquitin can form polymers in homotypic (the chain contains only one type of linkage) or heterotypic (chains with different types of linkages) ways. Heterotypic chains can be branched when ubiquitin is modified on two or more sides[37] (Figure 1.4).

The assembly of the different chain types can be oriented by E2s or E3s. In the case of the RING or U-box domain containing E3 ligases, the linkage specificity is most likely determined by the E2s. For instance the SCF ligases use the Lys48-specific E2 Ube2R1 to Lys48-polyubiquitinate substrates[38], while the anaphase-promoting complex (APC/C) uses the Lys11-specific Ube2S to produce Lys11-linked chains[39]. Some E3s can synthetize different chains depending on the E2 enzymes they pair up with: for example BRCA1-BARD1 assembles Lys48 linkages with Ube2K, but Lys63 linkages when bound to Ube2N-Uev1A[40]. On the contrary, HECT-type E3 ligases display linkage specificity. NEDD4 synthesises Lys63-linked chains, while E6-AP assembles Lys48-linked chains[41].

The most studied linkage types are Lys48 and Lys63. Lys48 chains play role in the regulation of proteasomal degradation. This is the only essential lysine on ubiquitin in yeast[42], and the most abundant linkage type in all tested organisms[43, 44]. Lys63-linked chains can trigger proteolysis by targeting substrates to lysosomes[45]. However, they have non-proteolytic functions as well, such as roles in trafficking, signaling, DNA damage response and in the immune system (reviewed in[46]). Roles of the other linkages are starting to emerge: Lys11 polyubiquitin chains are linked to proteasomal degradation of specific proteins involved in cell cycle progression[47]. The abundance of this type of linkage increases when the APC/C anaphase-promoting complex (or cyclosome) is active[48]. Lys11 chains might be involved in the NF-kB signaling pathway, as c-IAP1 and UbcH5 have been shown to assemble Lys11-linked ubiquitin chains on RIP1 in the TNFR1 complex [49]. The BRCA1- BARD1 complex was reported to assemble Lys6 linked chains on itself that can be recognized by RAP80, linking this type of modification to DNA repair and DNA damage response[50]. Lys29 linkages are enriched following proteasome inhibition[44, 51], and are formed on substrates of the ubiquitin-fusion degradation pathway[52]. Lys27 and Lys33 may be

assembled during stress response by U-box E3 ligases[53]. Lys27 chains were linked to mitochondrial biology, since this type of polyubiquitination occurs on several mitochondrial proteins by the E3 ligase parkin after mitochondrial damage[54]. In addition, Lys33 and Lys29 have been reported to inhibit kinase activity of members of the AMPK (AMP-activated protein kinase)-related protein kinases[55].

Additionally, the C-terminal glycine residue (Gly76) of ubiquitin can be linked on Met1 of the distal ubiquitin, forming linear chains. So far only one E3 ligase complex, the linear ubiquitin chain assembly complex (LUBAC) has been shown to assemble such chains[56]. Met1-linked chains play crucial roles in NF-kB signaling[57-59].

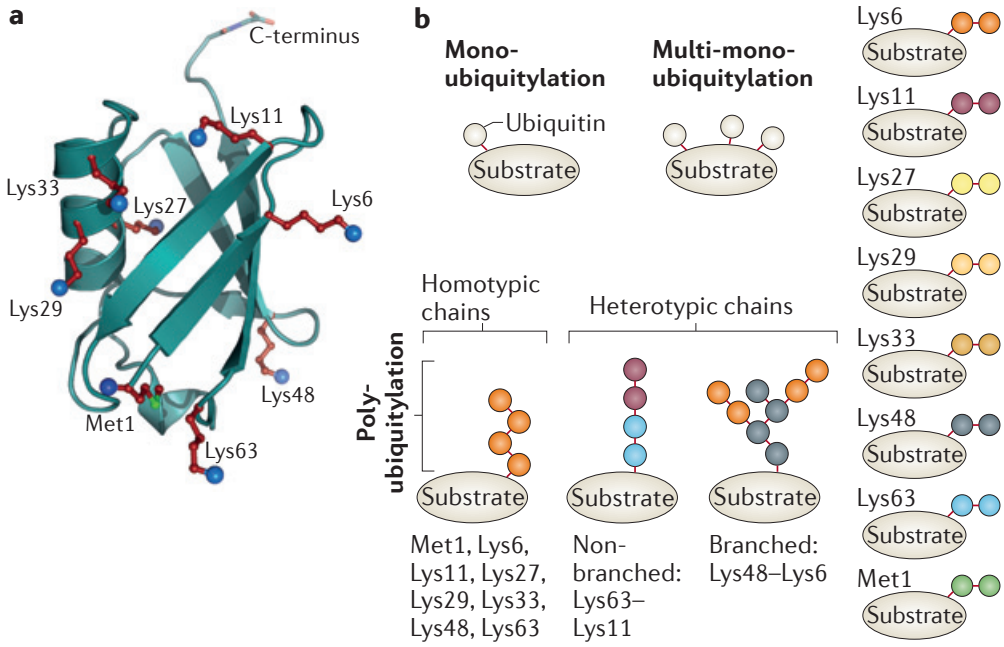


Figure 1.4: Lysines on ubiquitin and ubiquitin linkage types.

a, The seven lysine residues, Met1 and the C-terminus are shown in stick representation on the ubiquitin structure. Blue spheres indicate the amino groups that are modified during ubiquitin chain formation. b, Ubiquitin can modify substrates as a monomer or polymer of different linkage types. Adapted from:[60]

The signals created by ubiquitin chains are decoded by specific receptors in the cells that contain ubiquitin-binding domains (UBDs). UBDs bind non-covalently to ubiquitin signals. The interaction is linkage specific and directs proteins to cellular fates such as degradation or translocation. Linkage specificity of the domains is achieved by either the recognition of the linker region between two ubiquitin moieties or the detection of the spatial distribution and positioning of the units of the ubiquitin chain[61]. Most of the UBDs bind to the hydrophobic patch around Ile44 on ubiquitin (Leu8, Ile44, Val70); therefore this patch is essential for signalling. To what extend this patch is exposed depends on the type of the chain conformation that is determined by the linkages: Lys63-linked chains adopt an extended structure that exposes the patches, whereas Lys48 chains are in a compact conformation in solution, therefore the hydrophobic patch is buried. However, under physiological conditions this conformation oscillates between the open and packed structure[62]. Structural work has revealed that UBD binding to ubiquitin changes the conformation of ubiquitin[63]. The length of the chain is also an important determinant of the UBD-ubiquitin interaction: it has been shown that at least 4 Lys48-linked ubiquitin moieties are required for recognition by receptors associated with the 26S proteasome[64]. Binding affinities of individual UBDs for ubiquitin are generally low. Proteins containing multiple UBDs or multiple ubiquitin-binding surfaces in single domains make high-avidity interactions possible[65].

Several distinct UBDs have been described so far that are different in structures but non-covalently bind to ubiquitin. The most commons contain a single or multiple α-helices in their structure. This fold can be found for example in the ubiquitin-associated domains (UBAs) that are present in several proteins involved in ubiquitin-mediated proteolysis[66], such as in shuttle factors that deliver ubiquitinated substrates to the 26S proteasome (Figure 1.5). Shuttle proteins include HHR23A/B and PLIC1-4 (Dsk2 in

yeast)[67]. UBA domains generally bind Lys48-linked chains in strong preference to monoubiquitin[68]. The ubiquitin interacting motif (UIM) also folds into α–helical structures. It was first identified in an intrinsic ubiquitin receptor of the 26S proteasome, Rpn10/S5a[69]. Another types of UBDs contain zinc finger folds to engage in ubiquitin binding. The ubiquitin-binding zinc finger (UBZ) domains have been found in many proteins regulating DNA repair and have been shown to be crucial for recruiting proteins to the sites of DNA damage[70]. For example, PCNA monoubiquitination in response to DNA damage recruits the Y family DNA polymerases that recognize ubiquitin through their UBZ domains. This provides a high-affinity interaction and is essential for the successful repair of the damaged DNA[71] The pleckstrin-homology (PH) fold (such as the pleckstrin-like receptor for ubiquitin (PRU)) can be found in the other intrinsic proteasomal ubiquitin receptor, Rpn13[72]. E2 and E3 enzymes also have to bind to ubiquitin through their UBDs to fulfil their functions. The Ile36/ Leu71/Leu73 patch on ubiquitin has been described to be important for the interaction with the E2/E3 complex[73].

The so-called UBL (ubiquitin-like) fold can be also found in several proteins, including ubiquitin-like modifiers, or as a part of a multidomain structure in proteins that cannot be conjugated to substrates. Protein shuttle factors have UBL domains through which they can interact with the 26S proteasome[67] (see also Figure 1.5). Additionally, USP enzymes and E3 ligases have been predicted to possess such folds[74].

UBDs themselves can be regulated by posttranslational modifications: monoubiqitination of Rpn10 controls the receptor availability to bind ubiquitinated proteins[75]; and phosphorylation of the UBA domain of p62 enhances its binding to Lys63-linked chains and in turn regulates autophagy[76].

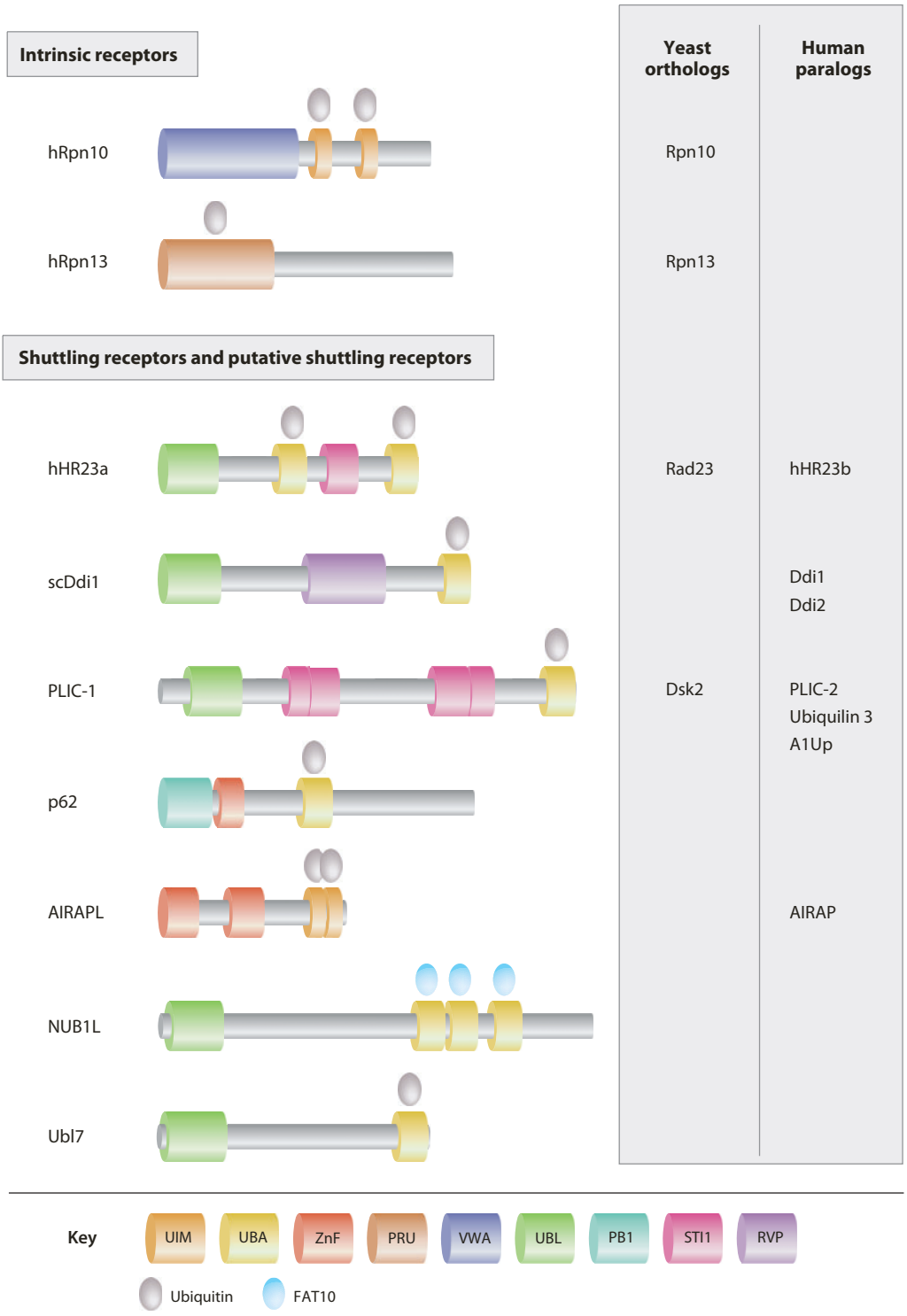


Figure 1.5: Examples of UBD-containing proteins and their domain structures.

The figures depict the human forms of the proteins, except for Ddi1. The Saccharomyces cerevisiae orthologs and human paralogs of the ubiquitin receptors are indicated in the grey table. 'Intrinsic' receptors indicate authentic proteasome receptors, while 'shuttling' receptors are reversibly associated with the proteasome. Apart from ubiquitin, all proteins and domains are drawn to scale. Adapted from: [77]

1.4. The 26S proteasome

The 26S proteasome is an over 2.5 MegaDaltons machinery that degrades proteins that are labelled with ubiquitin in an ATP-dependent manner[64]. The 26S proteasome is localized in the nucleus and the cytoplasm, and mainly degrades substrates processively. In some cases however, regulatory proteins are activated by the proteasome through the removal of inhibitory domains, a process called partial proteolysis[77]. 26S proteasomes are composed of a regulatory subunit (RP, 19S particle) and a core particle (CP, 20S particle) that is responsible for proteolysis (Figure 1.6). The CP has a barrel-like structure and its 28-subunits are arranged into four heteroheptameric rings[78]. The RP controls substrate entry to the CP by opening the substrate translocation channel. It consists of a lid and a base part. Since substrate degradation by the proteasome is ATP-dependent, six ATPase subunits are located on the base and help unfold the proteins to direct them to the CP through the translocation channel[79]. Receptors that recognize ubiquitin are associated with the lid: Rpn10 and Rpn13 are intrinsic subunits; Rad23, Dsk2 and Ddi1 are proteasome-associated proteins. The latter proteins contact the proteasome through their UBL (ubiquitin-like) domain, and their function is to shuttle polyubiquitinated proteins to the proteolytic complex[77]. Rpn10 is the first identified and best-characterized ubiquitin receptor, and is essential in mice. It recognizes ubiquitin through its two UIMs at the C-terminus[80], and has a VWA (von Willebrand A) domain on the N-terminus that is important for proteasome structure. hRpn13 is a ~42 kDa protein with an N-terminal Pru domain and a C-terminal domain that serves as the receptor site for the proteasome-associated DUB, Uch37[77].

Through the action of the RP and CP particles, proteins are hydrolysed into peptides that can be presented on the cell surface in complex with the major histocompatibility (MHC) class I molecule[81]. Therefore the proteasome plays role in adaptive immunity.

Alternatively, peptides can be further degraded to single amino acids by cytosolic peptidases.

The proteasome also contains deubiquitinating enzymes (DUBs) that cleave ubiquitin from substrates, the process required for effective degradation. The function of the Rpn11 DUB is critical for proteasome function in human and yeast[82]. Once proteins "committed" for degradation, Rpn11 removes chains at once due to its proximal specificity.

Apart from Rpn11, two other deubiquitinating enzymes are located on the RP: Usp14 (Ubp6 in yeast) and Uch37. These DUBs can trim ubiquitin chains prior to the commitment for degradation; therefore the substrate can be rescued from the proteasome. Their function has been proposed to be to suppress the breakdown of lightly ubiquitinated proteins[83]. Ubp6 has also been shown to play an important role in the ubiquitin homeostasis. In its absence, ubiquitin is rapidly depleted[84]. In yeast, free ubiquitin depletion induces the *UBP6* gene that in turn results in increased ubiquitin recycling[85].

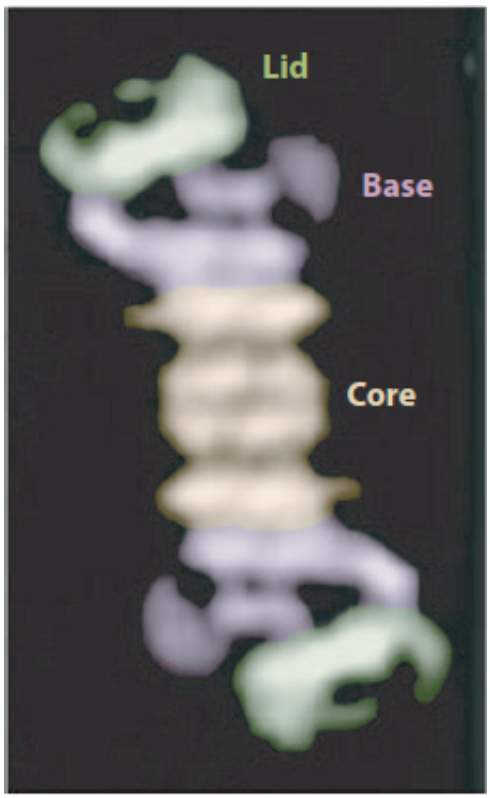


Figure 1.6: Structure of the proteasome holoenzyme.

The core, base and lid particles of the 26S proteasome are indicated. The image was generated by averaging of electron micrographs of negatively stained *Xenopus laevis* proteasomes. Adapted from[77]

1.5. NEDD8 and the NEDDylation pathway

NEDD8 (Neural Precursor Cell-Expressed Developmentally Downregulated-8) was first identified in the early 1990s among other genes that are downregulated in neural precursor cells during mouse brain development[86]. It is highly conserved in all studied eukaryotes (Figure 1.7), and essential for the viability of *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila*, *Arabidopsis* and mouse[87].

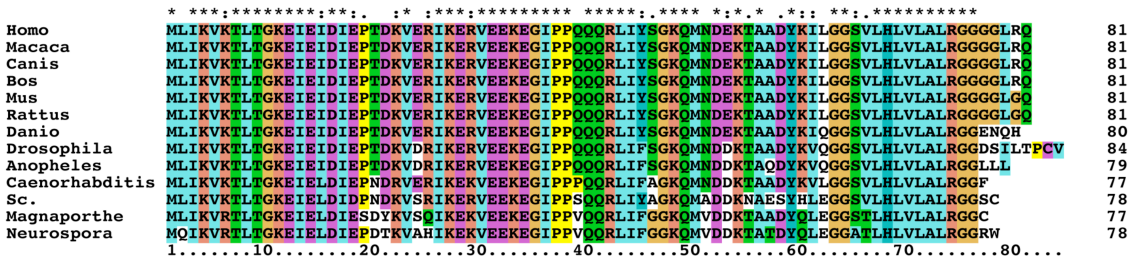


Figure 1.7: NEDD8 is highly conserved through species

Sequence alignment between *Homo sapiens* NEDD8 (accession number: NP_006147), *Macaca mulatta* (XP_001113390.1), *Canis lupus familiaris* (XP_537389.2), Bos taurus (NP_777189.1), *Mus musculus* (NP_032709.1), *Rattus norvegicus* (NP_620233.1), *Danio rerio* (NP_001002557.1), *Drosophila melanogaster* (NP_609919.1), *Anopheles gambiae str. PEST* (XP_317573.3), *Caenorhabditis elegans* (NP_492717.1), *Schizosaccharomyces pombe* (NP_595955.1), *Magnaporthe oryzae* (XP_365042.2), *Neurospora crassa* (XP_964486.1) was performed using ClustalX 2.0.12 software.

An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties. A . (period) indicates conservation between groups of weakly similar properties. The residues are coloured according to their physicochemical properties (red: small + hydrophobic; blue: acidic; magenta: basic; green: hydroxyl + sulfhydryl + amine + G; grey: others).

1.5.1. NEDD8 conjugation

NEDD8 is 80% homologues and 60% identical to ubiquitin; therefore it is the closest relative to ubiquitin among the ubiquitin-like molecules (ubls). NEDD8 is attached to its substrates in a similar manner to ubiquitination, but historically, NEDDylation is thought to be catalysed by a unique set of enzymes (Figure 1.8).

NEDD8 is synthetized as a precursor that needs to be cleaved at the C-terminal in order to expose the diglycine motif, through which it is covalently attached to substrate lysine residues. NEDP1 (also called SENP8 due to its sequence similarity to SUMO-specific proteases) has been shown to catalyse the maturation step with high specificity towards NEDD8[88-90]. It can also remove NEDD8 from substrates, the process called deNEDDylation. Additionally, a ubiquitin C-terminal hydrolase, UCH-L3 has been reported to process NEDD8 as well as ubiquitin[91, 92].

The NEDD8 E1 and E2s (Ubc12 and Ube2F) are specific for NEDD8 conjugation and distinct from the ubiquitin activating and conjugating enzymes[93, 94]. The NEDD8 activating enzyme (NAE) first adenylates the C-terminal glycine of NEDD8 in an ATP-dependent manner, and transfers it to the catalytic cysteine to form a thiolester bond. NAE is a heterodimer, composed of APPBP1 and Uba3 that are similar to the N-terminal and the C-terminal part of the ubiquitin E1, respectively[95-97]. The selectivity of the NEDD8 E1 that prevents misactivation of ubiquitin is determined by arginine 190 in Uba3 and alanine 72 in NEDD8 (arginine in ubiquitin)[97]. After activation, NEDD8 is transferred onto E2 enzymes, forming another thiolester bond. Ubc12 and Ube2F are E2 enzymes that contain a unique N-terminal domain that specifically docks within a conserved groove in Uba3 that is not found in other E1 enzymes. Therefore, it ensures optimal and specific transfer of NEDD8 from E1 to E2[98]. Although there is a

remarkable selectivity at the E1 and E2 level for NEDD8 activation and transfer, redundancy for E3-ligases and deconjugating enzymes has been observed. Dcn1 is a specific E3-ligase for NEDD8 conjugation[99-101], whereas Mdm2, c-Cbl and IAPs can conjugate both ubiquitin and NEDD8[102-104]. Other NEDD8 E3 ligases are closely associated with CRL functions, such as Rbx1 and Rbx2, and SCF^{FBX011}[105].

There have been a few studies suggesting that NEDD8 can form chains *in vivo* on Lys11, Lys22, Lys48 and Lys60[106, 107], These studies mainly used a mass-spectrometry-based approach to identify diglycine remnants on NEDD8 peptides after tryptic digestion, that is a sign of ubiquitin or NEDD8 chain formation (the caveats of this approach is discussed in Chapter 4.1). NEDD8 chains have been shown to build up on the catalytic cysteine of Ubc12 prior to conjugation to Cul1[108].

NEDD8 can be removed from its targets by deconjugating enzymes such as the NEDP1/SENP8/DEN1 protease and the COP9 signalosome (CSN)[91, 92]. The catalytic component of the 8-enzyme-complex CSN is CSN5 that is a zinc metalloprotease with high affinity towards NEDDylated cullins[109]. Overexpression of NEDP1 leads to deNEDDylation of cullins but endogenous NEDP1 is inefficient in cullin deNEDDylation and probably involved in NEDD8 cleavage from non-cullin substrates (discussed in details in Chapter 5.1). Other proteases, such as UCH-L3, UCH-L1[110], Ataxin-3[111] and PfUCH54[112] show dual activity for NEDD8 and ubiquitin. Usp21 has been also shown to be an isopeptidase for NEDD8[113], however, a recent study identified residues on NEDD8 that preclude interaction with this protease[114].

The NEDD8 ultimate baster 1 (NUB1) has been shown to bind NEDD8 and target NEDDylated substrates to degradation by the 26S proteasome. It has been reported to interact with the proteasomal subunit S5a[115, 116]. It possesses a UBL (ubiquitin-like)

domain at the N-terminal region and two UBAs (ubiquitin-associated domain) at the C-terminal region. It is induced by interferons and mainly localizes in the nucleus. NUB1 overexpression has been shown to lead to reduction of NEDD8 monomers and conjugates[117].

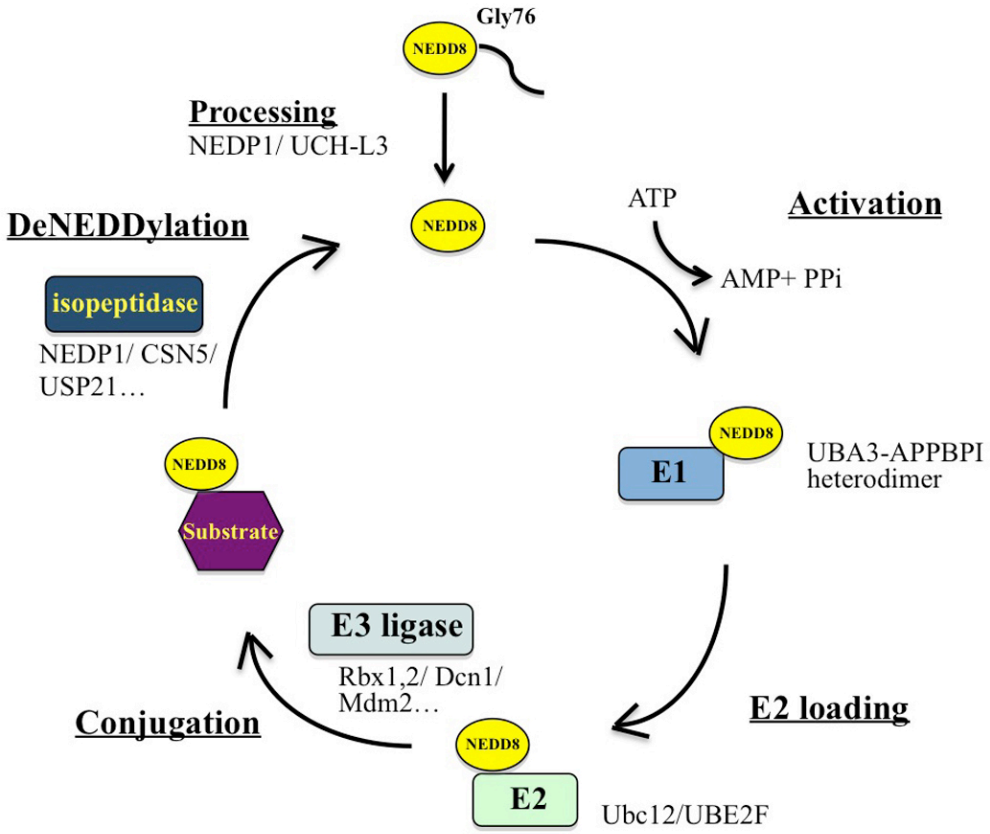


Figure 1.8: The NEDD8 conjugation cascade

NEDD8 processing from the precursors is catalyzed by NEDP1 or UCH-L3. NEDD8 is activated by the hetero dimeric E1 APPBP1-UBA3, then transferred onto the catalytic cysteine of Ubc12 or UBE2F, the NEDD8-specific E2 conjugating enzymes. E3 ligases catalyze the final step of NEDDylation. NEDD8 can be removed from substrates through the action of deNEDDylating enzymes.

1.5.2. Biological consequences of NEDDylation

The first identified substrate for Rub1 (the NEDD8 ortholog in yeast) was cdc53, a component of SCF E3 ligase complex in *Saccharomyces cerevisiae*[95, 118] and Cul4A in humans[119]. However, deletion of *Rub1* in *cerevisiae* does not cause lethality, contrary to other organisms, such as *S. pombe, D. melanogaster* and mice, where NEDD8 is required for viability. The inactivation of the temperature-sensitive NAE gene in the ts41 CHO cell line has been shown to cause cell cycle arrest at S-phase[120]. In *Arabidopsis thaliana*, downregulation of the NEDD8 pathway results in reduced auxin response[121]; whereas RNAi knockdown of NED-8 or enzymes of the conjugation pathway in *C. elegans* causes developmental abnormalities[122, 123] and hypersensitivity to ENU-induced germ cell apoptosis[124].

The **cullin** family of proteins are structurally related, and function as molecular scaffolds in assembling the Cullin RING Ligases (CRLs). CRLs are multi-component ubiquitin E3 ligase complexes, consisting of a cullin scaffold and Rbx1 or Rbx2, the catalytic RING subunits[125]. The members of the family are Cul1, 2, 3, 4A, 4B, 5, 7 and PARC and Apc2 in human cells, however, only Cul1-5 assemble CRL complexes[126]. Substrate recognition requires the assembly of the cullins with their own dedicated substrate receptor proteins, through an adaptor protein (Figure 1.9). The receptors bind to specific sequences of the substrates.

The CRL1 is also referred to as SCF ligase complex. It consists of Cul1, Rbx1 RING subunit; the adaptor protein Skp1 and a substrate receptor F-box protein. Skp1 is docked to Cul1 near to its N-terminus, while Rbx1 is on the C-terminal domain. Structural studies have shown similar architectures in the case of CRL2 and CRL5. They share a common adaptor: Elongin BC, through which substrate receptors such as the von Hippel-Lindau (VHL) tumor suppressor are recruited. CRL4A utilizes the 127 kDa

DDB1 (damaged DNA binding protein 1) as its adaptor, and DCAFs (DDB1 and Cul4A associated factors) or DWDs (DDB1-binding WD40 proteins) as substrate receptors. The BTB domain proteins have been shown to be both adaptors and substrate recognition modules for CRL3[127].

CRLs are regulated through different mechanisms, including NEDDylation on a conserved lysine near the C-terminus of cullins. NEDD8 modification stimulates the ligase activity by a) enhancing binding to the ubiquitin-loaded E2, b) enhancing ubiquitin transfer from the catalytic cysteine of the E2 to the substrate, c) positioning the E2 active site adjacent to the substrate, d) precluding binding of the cullin inhibitor CAND1[128]. Additionally, NEDD8 modification of Cul3 has been shown to promote dimerization of the scaffold proteins (where NEDD8 covalently modifies one Cul3 molecule while non-covalently interacts with the second one)[129]. Rbx1 and Ubc12 NEDDylate Cul1, 2, 3 and 4, whereas Cul5 NEDDylation is mediated by Rbx2 and Ube2F[130]. Furthermore, Dcn1 has been shown to enhance cullin NEDDylation[131]. DeNEDDylation of cullins occurs through the action of the COP9 signalosome (CSN). The catalytic component of the 8-subunit enzyme complex is CSN5, a JAMM-metalloproteinase. Interestingly, the COP9 signalosome structurally resembles the proteasome lid. The enzymatic activity of CSN is also analogous to that of the lid, and Csn5, like Rpn11, carries the metalloprotease active site[132]. There have been studies suggesting that the lid can be replaced by CSN[133, 134].

In the absence of NEDD8 modification, CAND1 (Cullin-Associated and Neddylation-Dissociated 1) binds to cullins and inhibits their activity[135]. The C-terminus of the bound CAND1 excludes adaptor and substrate receptor protein binding, while the N-terminus binds to the NEDD8 acceptor lysine[105].

A recent study using the NEDD8 E1 inhibitor MLN4924 has demonstrated in a quantitative proteomics approach that most of the cullins bind to adaptors, and the abundance of the adaptors determine CRL activity, rather than their NEDDylated status or the binding of CAND1[136]. It has been also observed in several studies that CAND1 is required for intact CRL activity[137, 138]. Recent data from Raymond Deshaies and Dieter Wolf group seem to solve the CAND1 paradox: they have shown that CAND1 is responsible for Cul1-Fbox disassembly for the rearrangement of the new SCF complex. This process is controlled by cullin deNEDDylation by CSN5, an event regulated by the presence or absence of substrate (ZOMES VII meeting, 2012, oral presentation).

Interestingly, a bacterial mechanism for inhibiting NEDD8 conjugation has been shown recently: CHBP or Cif, the bacterial effector deamidase promotes deamidation of Gln40 of NEDD8 that results in decreased CRL activity[139]. This is an interesting example how pathogens can target ubiquitin-like conjugation pathways and could be important for drug development.

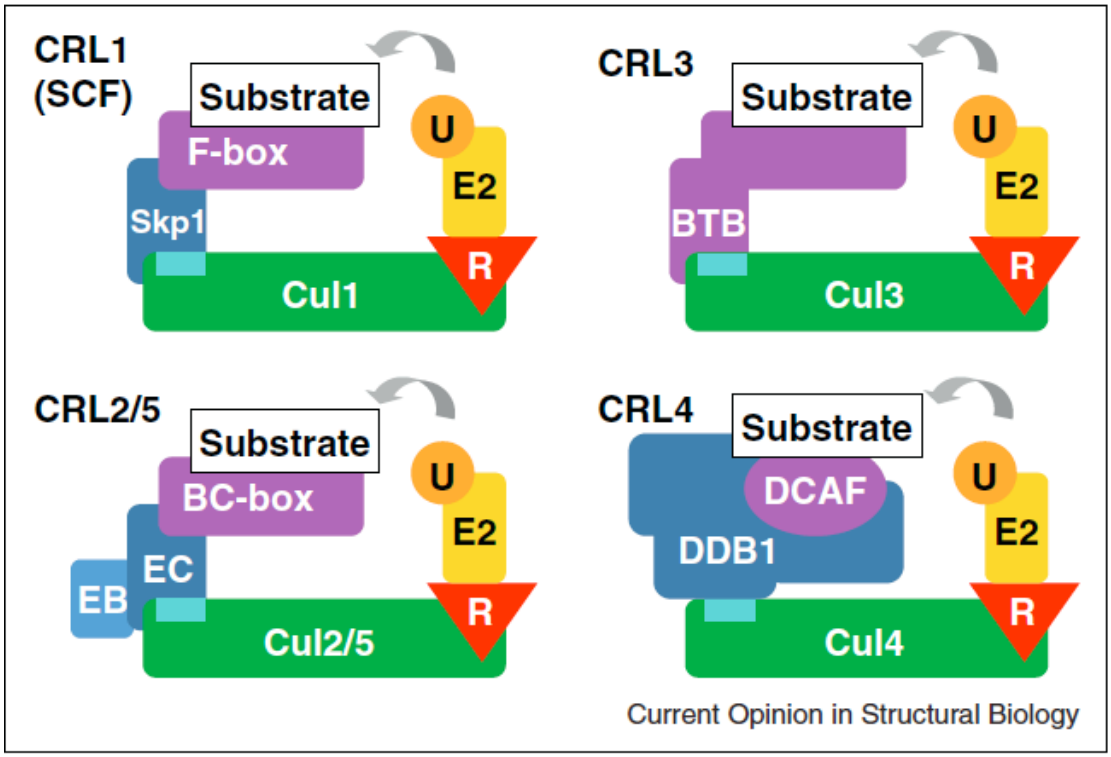


Figure 1.9: Composition of the Cullin RING E3 Ligases.

Adapted from: [105]. Cul1-5 (green) are in complex with Rbx1/2 (red). Adaptors are shown in light blue, while substrate receptors are highlighted in magenta color. The ubiquitin is presented in orange, while the E2 conjugating enzyme in yellow color.

Other important substrates for NEDD8 are the tumor suppressor **p53**, and its main regulator, the ubiquitin and NEDD8 E3 ligase **Mdm2**. NEDD8 modification on p53 increases its stability, and in addition it inhibits its transcriptional activity. Mdm2 itself is NEDDylated, and the modification significantly increases its protein stability[102]. p53 NEDDylation can be also mediated by FBX011, a member of the F-box protein family and a component of the SCF (Skp1.Cullin1.F-box) complex[140]. p53 fused with NEDD8 at the C-terminus has been found exclusively in the nucleus[141]. NUB1, a NEDD8 interacting protein was shown to inhibit p53 NEDDylation and stimulate mono-ubiquitination, resulting in nuclear export of the tumor suppressor[142]. A regulator of the p53-Mdm2 pathway, Tip60 acetyltransferase was shown to

preferentially inhibit Mdm2-mediated p53 NEDDylation, but not ubiquitination[143], and this activity was related to the ability of Tip60 to relocalise p53 to PML bodies.

Mdm2 also mediates NEDDylation of **TAp73** (full-length p73), resulting in the cytoplasmic localization of the protein. Transcriptional activity of TAp73 has been shown to increase in the ts41 CHO cell line at the non-permissive temperature, indicating that NEDDylation inhibits its transcriptional activity[144].

VHL (von Hippel-Lindau) protein, a component of the Cul2-based CRL has been also shown to be NEDDylated, and the modification prevents its interaction with cullin 2 and promotes VHL association with fibronectin. Point mutations that prevent NEDDylation of VHL are associated with von Hippel-Lindau disease and lung cancer[145].

NEDDylation has been shown to increase the efficiency of **EGFR** ubiquitination by the shared NEDD8 and ubiquitin E3 ligase, c-CBL, and facilitates its lysosomal degradation[103].

Endogenous breast cancer-associated protein 3 (**BCA3**) has been shown to be modified by NEDD8 on multiple lysine residues. Moreover, NEDP1 knockdown increased BCA3 NEDDylation, indicating that the deNEDDylating enzyme regulates the protein *in vivo*. Modification of BCA3 by NEDD8 promotes SIRT1 recruitment and therefore suppresses NF-κB-dependent transcription[146].

Ribosomal proteins are a large class of NEDD8 substrates. NEDDylation of L11 is required for its stability, and it also regulates its localization. In the event of nucleolar stress, caused by low doses of Actinomycin D (which preferentially inhibit RNA Polymerase I and rRNA production), L11 relocalises to the nucleoplasm. The translocation results in binding to Mdm2, repression of Mdm2-mediated ubiquitination

of p53, and leads to increased level of the tumor suppressor protein and cell cycle arrest[147]. It has been shown that the NEDDylated form of L11 localises in the nucleolus. Upon low doses of ActD treatment, NEDDylation of L11 decreases, causing relocalization of L11 from the nucleolus to the nucleoplasm, which provides a signal for p53 activation[148].

PINK1 and parkin have been recently found as novel NEDD8 targets. The modification results in increased parkin E3 ligase activity and stabilized PINK1[149].

Recently, NEDD8 has been reported to regulate the transcription factor **E2F-1**, in a manner analogous to p53 regulation. One study showed that the modification increases E2F-1 stability, decreases its transcriptional activity and slows cell growth[150]. Another group reported that NEDDylation of E2F-1 determines its target specificity and might play important role in promoting apoptosis upon DNA damage[151].

Additional substrates for NEDD8 that play role in DNA repair, chromatin remodeling, replication and mRNA splicing have been identified by proteomics approaches[106]. Further characterizations of these proteins are needed to specify the role of NEDD8 modification in their regulation.

1.6. Ubiquitin-like modifiers

Apart from ubiquitin, other polypeptides have been identified to regulate proteins through covalent modification of their lysine residues. These ubiquitin-like modifiers are attached to substrates in a reversible manner, through an enzymatic cascade that is distinct but evolutionally related to ubiquitination (Figure 1.11).

Ubiquitin and ubls share the same three-dimensional structure, the β-grasp fold (Figure 1.10). Although ubiquitin and ubls cannot be found in prokaryotes (the only exception

might be Pup), several proteins adopt the ubiquitin-fold, including MoaD and ThiS that are involved in molybdopterin and thiamin cofactor biosynthesis, respectively. The activation of MoaD and ThiS is also strikingly similar to that of the ubls, except that it results in the attachment of a sulphur atom to their C termini, and not their attachment to a substrate.[152].

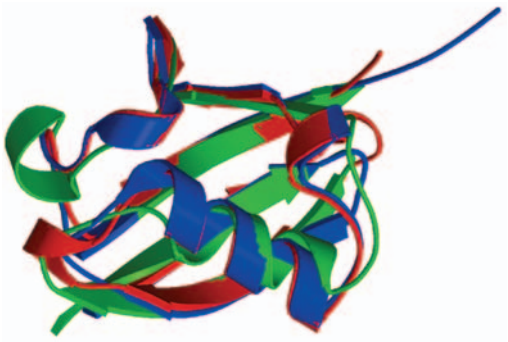


Figure 1.10: The ubiquitin superfold.

The figure shows an overlay between ubiquitin (blue), SUMO (green) and NEDD8 (red). Adapted from:[153]

An interferon-induced gene product, **ISG15** was the first ubl discovered, four years after the discovery of ubiquitin. Its primary sequence consists of 2 domains that share significant homology with ubiquitin. The components of the ISG15 conjugation pathway are also induced by interferons. UBE1L is a specific E1 for ISG15, and UBCH8 is a shared E2 with ubiquitin. A few E3s have been identified for ISG15, including estrogen-responsive finger protein (RING-type) and Herc5. USP18 has been shown to reverse ISGylation.

Hundreds of proteins have been identified as targets for ISG15. In a recent study, a proteomics approach has revealed that ISG15 conjugation is restricted to newly synthesised proteins, including newly translated viral proteins in infected cells, causing disruption in viral particle assembly[154].

The small ubiquitin-like modifier **SUMO** family includes three members, SUMO-1, -2 and -3. They share a similar three-dimensional structure, but only approximately 20% sequence identity with ubiquitin. SUMO-2 and -3 only differ by three amino acids in their mature form and they are only 50% identical in sequence to SUMO-1[155]. SUMO is activated by a heterodimeric E1, and conjugated through the action of the E2 enzyme Ubc9 and E3 ligases, including RanBP2, PIAS family members, Pc2 and Topors[156, 157]. SUMOylation is a reversible modification, SUMO-specific proteases include SENP1, SENP2, SENP3, SENP5 and SENP7[158].

The first identified role for SUMO-1 was the modification of RanGap1 that targets it to the nuclear pore[159, 160]. Since then, SUMOs have been shown to play role in diverse stress responses, including heat shock, osmotic and oxidative stress[161]. SUMO 2/3 have been shown to form chains on target proteins that recruit SIM (SUMO interacting motif)-containing effector proteins, providing important regulatory mechanisms[162]. Poly- SUMO chains can act as a signal for recognition by ubiquitin E3 ligases to target the substrates for proteasomal degradation. An important example is the degradation of the PML protein. PML was identified in promyelocytic leukemia to be fused to retinoic acid receptor-α (RARα) and dispersed into small nuclear bodies. SUMOylation of PML recruits the SIM-containing ubiquitin E3 ligase, RNF4 that subsequently ubiquitinates the fusion and targets it to degradation[163]. Additional roles for SUMOylation include regulation of transcription[164], nucleo-cytoplasmic transport, neuronal survival[165], DNA replication and repair[166], cancer development and cell cycle progression[157].

Urm1 (ubiquitin-related modifier 1) was discovered as a protein showing sequence similarity to the bacterial sulphur carriers ThiS and MoaD. It is activated by Uba4, a

Ube1 related protein[167, 168]; and modifies a handful of proteins identified in a recent proteomics study[169]. It does not only attach to proteins, but also acts as a sulphur carrier in tRNA thiolation[170].

FAT10, also called diubiquitin, contains two ubiquitin-like domains in tandem[171]. It is covalently attached to substrate lysine residues similarly to ubiquitination, and serves as a signal for proteasomal degradation. Its degradation is accelerated by interaction with NEDD8 ultimate buster-1L (NUB1L)[172]. NUB1L is a splice variant of NUB1 possessing an extra UBA domain, and it was first described as a NEDD8 interacting protein[115]. However, it has higher affinity towards FAT10 than NEDD8. FAT10 and ubiquitin share an E1 activating enzyme, Uba6. USE1 (UBA6-specific E2 enzyme) is a recently identified E2 for FAT10 that is self-FATylated[173]. E3 ligases for the modification still remain to be found. FAT10 covalently modifies p53[174] and the ubiquitin activating enzyme Ube1[175]. In a recent study, the autophagosomal receptor p62/SQSTM1 (p62) was also found to be a genuine substrate for the modification [176]. FAT10 is induced by the proinflammatory cytokines IFN-γ and TNF-α in most cell lines, and constitutively expressed in B cells and mature dendritic cells[177-179].

ATG8 and **ATG12** are induced by starvation, and are essential for autophagosome formation, hence their name: autophagy-related ubiquitin-like modifier. Autophagy mediates the degradation of long-lived proteins and protein aggregates through the formation of a double-membraned vesicle in the cytoplasm, followed by fusion with the lysosome and hydrolysis of its contents. ATG12 is activated and conjugated by the E1- and E2-like enzymes ATG7 and ATG10, respectively[180, 181]. ATG5 is a substrate for ATG12, and its modification has been shown to be required for the elongation of the

isolation membrane to form autophagosome.

During autophagy, ATG8 (LC3) is crucial for the so-called 'lipidation', autophagosome maturation. It is covalently conjugated to the common membrane phospholipid, phosphatidylethanolamine to promote membrane association of the protein[182]. Unlike ATG12, ATG8 needs to be processed by ATG4 to expose the C-terminal glycine residue. ATG8 shares the E1 enzyme ATG7 with ATG12. This was the first example of a shared activating enzyme between two ubiquitin-like modifiers. ATG3 is the E2 conjugating enzyme for ATG8, and ATG4 has been identified to be responsible for deconjugation as well[182].

UBL5 is an unconventional ubiquitin-like molecule. It is highly conserved, but poorly characterized in higher eukaryotes. Most of the functional studies have been done on the *Saccharomyces cereviseae* homolog, Hub1 (homologous to ubiquitin-1). Budding yeast Hub1 deletion mutants are viable, while the corresponding *Schizosaccharomyces pombe* mutant is lethal. Hub1 is not conjugated covalently to proteins, and possesses a C-terminal dityrosine (YY), instead of the diglycine[183, 184]. The role of Hub1 in alternative splicing has been recently demonstrated in yeast: it modifies the spliceosome in a way that enables it to use certain non-canonical 5' splice sites[185]. It has been also shown to be involved in the mitochondrial unfolded protein response[186], cell cycle progression and polarized growth[187].

Even though prokaryotes lack the ubiquitin proteasome system, *Mycobacterium tuberculosis* (Mtb) has analogous proteolytic machinery. **Pup** (prokaryotic ubiquitin-like protein) is a 64-residue protein that has been shown to modify proteins and to target them for degradation by the mycobacterial proteasome[188]. Pupylation involves the

action of an enzymatic cascade that is analogous to the ubiquitin E1-E2-E3, and proteins with 'depupylase' activity have been described as well[189, 190]. Pup is attached to substrate lysine residues through its C-terminal glutamate, which is converted from the original glutamine before substrate conjugation. The pupylation cascade and the Mtb proteasome itself are promising drug targets against the pathogen.

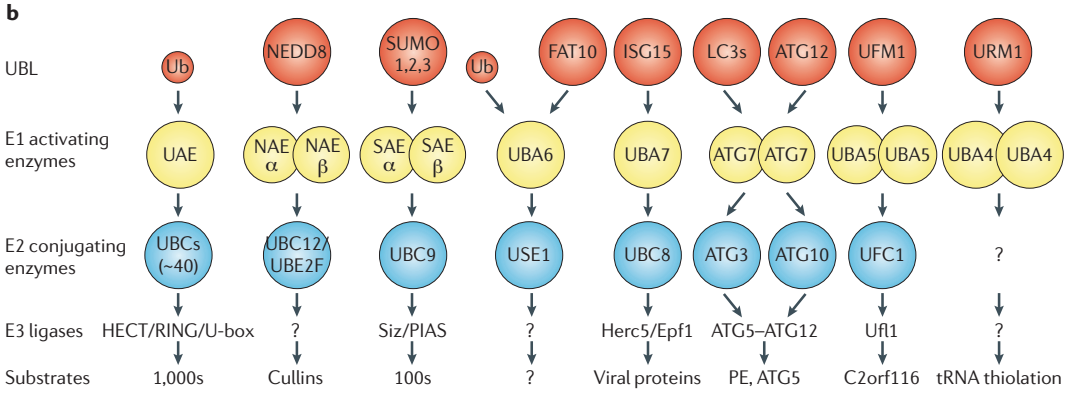


Figure 1.11: Ubiquitin-like molecules and their enzymes.

Nine classes of ubls are shown in the figure, each of which are conjugated through their cognate enzymatic cascade. Except for ubiquitin that can be activated by UAE (Ube1) and Uba6 as well. Adapted from[191].

Crosstalks between these modifications enhance the complexity of protein regulation by ubls. A single lysine residue can undergo different types of modifications; for example p53 and Mdm2 have similar lysine requirements for either ubiquitination, SUMOylation or NEDDylation[192]. Other proteins such as HIF-1α, IκBα, CREB, PCNA, and Huntingtin can be SUMO or ubiquitin modified with different biological outcomes[193]. An example is the coordination of DNA replication by SUMO and ubiquitin on the proliferating cell nuclear antigen (PCNA). Ubiquitination in response to

DNA damage is required for the bypass of replication-blocking lesions, while SUMO modification contributes to the Srs2 helicase recruitment to replication forks[194]. The earlier-mentioned PML degradation coordinated by sequential SUMO and ubiquitin modification is one of the several examples of close interaction between the pathways. Another crosstalk has been studied in great details is the activation of the Cullin RING ubiquitin E3 ligases by NEDD8. It has been also shown that proteins can be simultaneously modified by different ubls. Moreover, there are a few examples where ubls share E1, E2 or E3 enzymes that initially thought to be specific for a distinct ubl conjugation pathway. For instance, FAT10 is activated by the ubiquitin E1 enzyme Uba6; ISG15 shares an E2 enzyme, UBCH8 with ubiquitin; and NEDD8 conjugation can be coordinated by ubiquitin E3 ligases Mdm2, c-Cbl and XIAP.

1.7. Stress response

Cells have to maintain their viability under a wide variety of stressful conditions. Transcription, translation and protein degradation are all parts of the stress response, and dynamic protein regulation by the ubiquitin and ubiquitin-like pathways have been also shown to play important roles in cell survival.

1.7.1. Heat stress:

Cells sense elevated temperatures by proteins, since their proper structure and folding is only achieved in a small temperature range. A few degrees of difference from the optimal growth can result in unfolded and damaged proteins that are sensed by the cells. There have been many different ways evolved to react to these conditions: chaperons, heat shock proteins are induced to help proper refolding, or ultimately damaged proteins

are removed by different ways: proteases, lysosomes or the proteasome. Heat shock also results in damage of the structural parts of the cells: the cytoskeleton, microtubules etc. Elimination of aggregates and damaged proteins is essential for survival, and malfunctioning of this process is associated with disease and aging[195].

Bacteria respond to heat stress by inducing many genes that encode proteases that eventually degrade proteins[195]. In yeast several proteases and component of the UPS, including ubiquitin are also induced[196]. Apart from ubiquitin, the involvement of the small ubiquitin-like modifier in the heat shock response has been also well established. Increased SUMO attachment to targets after heat shock has been shown to help solubilize the proteins[197]. Moreover, the vast SUMO-2/3 conjugation in response to heat stress also defends cells from hyperthermic cytotoxicity[198].

1.7.2. Oxidative stress:

Cells are exposed to reactive oxygen-species (ROS) from environmental oxidizing agents and H_2O_2 that arise as a by-product of aerobic metabolism[199]. ROS exposure results in oxidization damage of proteins, DNA and lipids, and has been linked to aging and disease such as diabetes, neurodegeneration and cancer. In response to oxidizing agents, cells normally arrest and induce genes involved in restoring redox balance.

ROS has been shown to cause perturbation in the SUMO and the ubiquitin conjugation pathways. In the presence of H_2O_2, SUMO conjugates including modified transcription factors disappear, due to the inhibition of the conjugating enzymes: the SUMO E1 forms a disulfide bond with Ubc9[200]. In yeast, the ubiquitin E2 enzyme Cdc34 is sequestered through a disulfide complex with Uba1 in response to oxidation. This in turn causes stabilization of its target, Sic1 that results in a delay in cell cycle progression[201].

1.7.3. Ubiquitin stress:

An interesting paradigm is the so-called ubiquitin stress. In yeast, the sensitivity to many environmental conditions can be linked to the exhaustion of the ubiquitin pool. Such conditions include heat shock, cadmium, canavanine, cycloheximide, anisomycin, chloramphenicol, trichodermin, methotrexate, 4NQO, and methylmethanesulfonate[202, 203]. A low level of ubiquitin is dangerous since ubiquitination is critical in cell cycle regulation, DNA repair, protein sorting and quality control, apoptosis and other important processes.

1.8. Ubiquitin and ubiquitin-like molecules in disease

The ubiquitin-proteasome system and ubl pathways are essential for the maintenance of cellular homeostasis. Their malfunctions are associated with numerous types of disease, including cancers, neurodegenerative diseases, heart failure and viral diseases. Due to the involvement of UPS and ubls in important regulatory processes, they have the potential to be targeted by small-molecule inhibitors that influence distinct proteins.

Several enzymes involved in ubiquitin and ubl conjugation are associated with disease (for examples, see Table 1.1). The development of drugs target different parts of the system. The least selective are the proteasomal inhibitors that block degradation of many proteins. Several studies indicate that inhibition of the proteasome influences a wide range of pathways that eventually lead to anti-tumor effects. In the absence of intact proteasome, apoptosis is upregulated, while factors of angiogenesis, growth and survival are downregulated[204] (Figure 1.12).

The first clinically validated drug was bortezomib (velcade), an inhibitor of the 26S proteasome for anticancer treatment, developed by Millenium Pharmaceuticals. It is in clinics to treat multiple myeloma and mantle cell lymphoma. It targets the 20S proteasome, and is slowly reversible[191]. Disruption of the NF-κB signaling pathway, activation of ER stress, hypoxic response deregulation and mTOR inhibition are some of the mechanisms by which bortezomib has been implicated in killing cancer cells.

Interestingly, there might be a new therapeutic application of bortezomib: initial experiments suggest that it could be used to deplete normal antibody-producing plasma cells in transplant patients to prevent from antibody-mediated rejection[205].

Second generation inhibitors for different parts of the proteasome are also being developed for greater drug pharmacology.

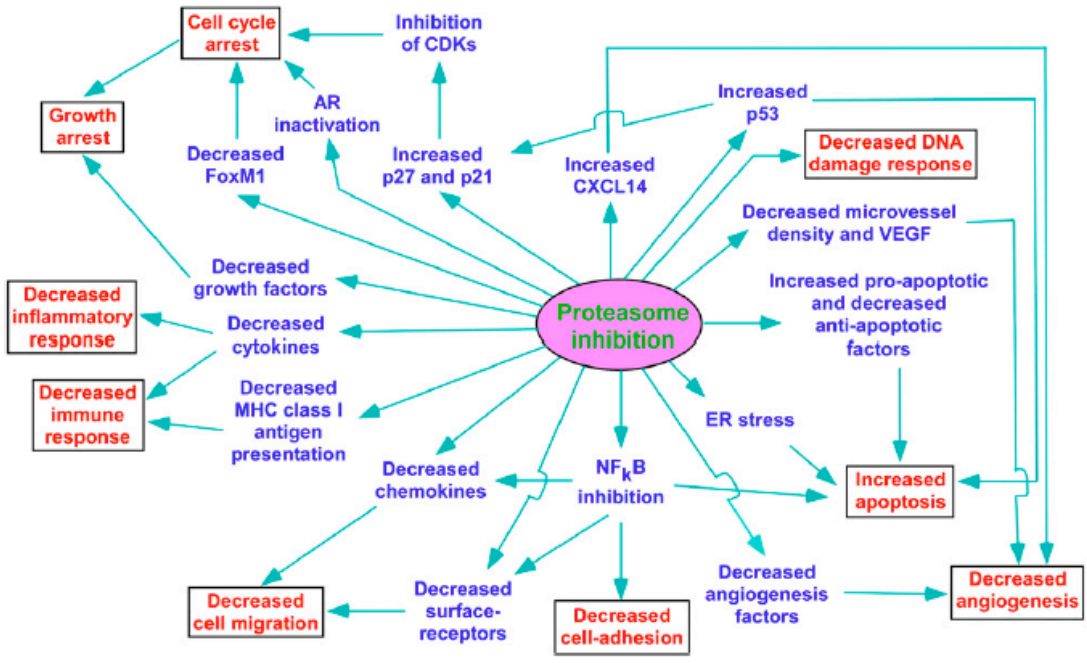


Figure 1.12: Effects of the proteasome inhibition.

The diagram is a schematic represenatation of the effect of proteasome inhibition on different pathways that contribute to cancer prevention. Adapted from: [204]

Several studies have linked the NEDD8 pathway to cancer. First of all, CRLs regulate the activity of proteins involved in cell cycle regulation and subsequently in tumorigenesis, and components of the ligase complex are often mutated, overexpressed or amplified in several cancers[206]. Elevated NEDD8 conjugation has been observed in oral squamous cell carcinoma and melanoma cell lines[207]. Among other substrates for NEDD8, tumor suppressors and oncoproteins can be found.

In order to block a specific ubl, the cognate E1 enzyme can be targeted. This results in specific inhibition of the ubl. The best example is MLN4924, the selective and potent inhibitor for the NAE[208]. It has also been developed at Millenium Pharmaceuticals, as a result of a screen for selective NAE inhibition. It is a competitive ATP inhibitor of NAE, an adenosine sulphamate analogue that forms a NEDD8-MLN4924 adduct and binds tightly to the nucleotide-binding site of Uba3, inhibiting its enzymatic activity. The covalent NEDD8-MLN4924 adduct is formed in situ through a mechanism called substrate assisted inhibition. First NEDD8-AMP is formed, followed by the thiolester bond between NEDD8 and the catalytic cysteine of NAE. MLN4924 binds to AMP binding site of the loaded NAE and attacks the thiolester bond between the Uba3 subunit and NEDD8, forming the adduct[209] (Figure 1.13.).

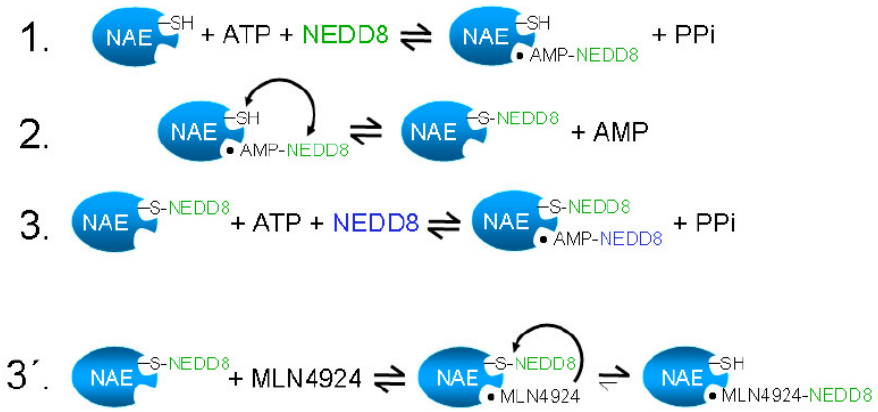


Figure 1.13: Schematic representation of the enzymatic mechanism of NAE-dependent NEDD8 activation and NEDD8-MLN4924 adduct formation.

Step 1: MgATP and NEDD8 bind to the enzyme. The reaction yields in a NEDD8-AMP formation and release of inorganic pyrophosphate (PP_i).

Step 2: NAE-NEDD8 thioester is formed and AMP is released.

Step 3: A second ATP and NEDD8 bind NAE and a second NEDD8-AMP is formed that occupies the adenylation domain of NAE. This form of NAE transthiolates NEDD8 to Ubc12.

Step 3': MLN4924 binds to the nucleotide binding pocket of the NAE-NEDD8 thioester form of NAE and forms a NEDD8-MLN4924 adduct with the thioester-bound NEDD8.

Adapted from: [209]

As a result of NAE inhibition, CRL activity is inhibited, and subsequent stabilization of the targets, eg. CDT1, p27 and NRF2 are achieved, upstream of the proteasome. MLN4924 is now in clinical phase II trial for the treatment of acute myeloid leukemia (AML), since CRL targets are essential for AML cell survival. In response to the treatment, AML cells undergo increased apoptosis mainly due to the disruption of

cellular redox status[210]. Since the range of NEDD8 substrates include tumor suppressors and oncogenes, MLN4924 may be a promising drug for other types of cancers too. Similar adducts to inhibit the ubiquitin- and SUMO activating enzymes are being tested to target specifically the ubl pathways.

Inhibition of the proteasome or E1 enzymes affect a wide range of substrates. On the other hand, E3s are thought to possess substrate specificity. Therefore screening for ligase inhibitors is a popular method for developing therapeutic agents. There have already been a few examples proven that some E3s are associated with disease, and they can be mechanistically targeted. Table 1.1 shows the most common E3s associated with disease and their inhibitors.

E3 ligase	Substrate	Disease association	Inhibitor/intervention investigated
HDM2	p53, p27	Breast and lung cancer, oesophageal carcinomas, glioblastomas and malignant melanomas	Nutlins and RITA promote p53 stabilization, Parthenolide promotes ubiquitination
$CRL^{SKP2/\beta TRCP/FBW7}$	p21, p27, cyclinD, β-catenin, IκBα	Multiple cancers and other disorders linked to the NF-κB pathway	NAE inhibitor
IAP	Substrates involved in apoptosis and signalling	Oesophageal, liver and lung cancers, ovarian carcinoma and MALT lymphoma	SMAC mimetics promote IAP degradation
E6-AP	p53	Cervical cancer; mutations associated with Angelman's syndrome	Expression of mRNA decay factor TTP stabilizes p53 through E6-AP targeting in HPV-transformed cervical cancer
VHL	HIF1α, HIF2α	VHL syndrome; renal cell carcinoma	Bioengineered VHL protein to increase HIF degradation
Parkin	Synphilin 1, Parkin	Parkinson's disease	Nitric oxide inhibits E3 activity

Table 1.1. Examples of E3 ligases associated with human disease.

Adapted and modified from:[191]

Although there are only about 100 DUBs identified so far, their biological roles make them attractive to further study them and develop inhibitors that can be applied in pharmaceutical interventions. Small molecule drugs have been suggested to be good

inhibitors for metalloproteases. However, in the case of the larger class of DUBs, the cysteine proteases, finding inhibitors that can be used as drugs has been problematic, since the currently available inhibitors do not get into the cells[191]. Regardless, there have been efforts to turn these inhibitors into drugs, because individual DUBs are associated with pathways involved in specific diseases, such as USP1 in the Fanconi anaemia DNA repair pathway[211] or USP7 with non-small cell lung adenocarcinoma[212]. Parkinson's disease was also linked to the loss of the deubiquitinase activity of UCH-L1[213].

Interestingly, bacteria and viruses have been also shown to interfere with the ubiquitin/ubl conjugation system on the DUB level. Host cell encoded proteases can be manipulated by pathogens during infection. Additionally, there are pathogen-encoded proteases with ubiquitin/ubl specific activities that target the host cells. Targeting these enzymes for therapeutic interventions is important, since many infectious diseases are still not curable (reviewed in [214]).

The ubiquitin-proteasome system plays a major role in degrading neuronal proteins. The malfunctioning of the UPS is associated with the accumulation of insoluble protein aggregates in neurons, the phenomenon observed in age-related and chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's, amyotrophic lateral sclerosis and dementia with Lewy bodies. Interestingly, polyubiquitin conjugates also accumulate in these disease conditions, and NEDDylation has been found to be deregulated in Alzheimer's disease[215, 216].

In Huntington's disease, the spinocerebellar ataxias can promote the formation of protein aggregates that are resistant to degradation by the proteasome and impair proteasome function[217]. Similarly in Alzheimer's disease, the formation of neurofibrillary tangles and plaques associated with amyloid-β protein aggregation and/or ubiquitinated TAU accumulation can impair proteasome function[218].

Ubiquitin and SUMO have been also linked to cardiovascular diseases[219]. For example, cardiomyopathies are associated with deregulated UPS functions and accumulation of ubiquitinated proteins[220]; and ischemic myocardial injury is linked to the deficiency of the ubiquitin ligase CHIP[221].

1.9. DNA damage, apoptosis and the regulation by the ubiquitin pathway

1.9.1. DNA damage and ubiquitin

DNA damage can result from stochastic errors in replication or recombination, endogenous reactive oxygen species or from environmental and therapeutic genotoxins. To ensure the integrity of genomic DNA and prevent tumor formation, the cell is able to detect the damage and signal to elicit cellular responses that include DNA repair, cell cycle arrest, senescence and apoptosis[222].

The ubiquitin pathway has been widely implicated in the various types of DNA damage responses. Recently, large-scale proteomics studies identified that many ubiquitination-mediated processes are engaged in promoting cellular responses to genotoxic stress[44, 51]. The most recent one quantified ~6,700 ubiquitination site changes in response to ultraviolet irradiation[223].

An example of the DNA damage regulation by the ubiquitin system is that the replication licensing factor Cdt1 has been shown to be regulated by the Cul4A-DDB1 ubiquitin ligase and by CRL1. Degradation failure of Cdt1 results in re-replication and G2/M DNA damage checkpoint activation[224, 225]. The NAE inhibitor MLN4924 has

been shown to induce apoptosis by blocking Cul4A NEDDylation that in turn deregulates Cdt1 turnover[226].

PCNA is involved in checkpoint control and DNA repair, and its roles are also regulated by its ubiquitination status. Monoubiquitination of the protein facilitates translesion synthesis, while polyubiquitination is required for postreplicational repair[222].

In addition, monoubiquitination of FANCD2 by UBE2T and the FA core complex is required for the intact DNA damage response by the Fanconi anemia pathway[227]. The modified form of the protein recruits the FAN1 DNA repair nuclease to DNA damage sites[228].

An important step of the DNA damage response is the recruitment of the ubiquitin E3 ligase RNF8 to DNA damage sites. The enzyme promotes the formation of Lys63 polyubiquitin chains on a target yet to be identified. This signal recruits RNF168 that promotes monoubiquitination of H2A and H2AX that is primed for Lys63 chain formation and recruitment of further effectors[229].

The SUMO pathway is also engaged in the DNA damage response. All three SUMO paralogs accumulate at DNA damage sites[230]. A recent example is the report on the SUMO-targeted ubiquitin E3 ligase RNF4 that is recruited to sites of damage by SUMOylated MDC1. The ligase recognizes the SUMOylated protein through its SUMO interacting motifs. Absence of RNF4 results in delayed clearance of DNA damage factors from the foci and defective loading of replication proteins[231].

1.9.2. Apoptosis

Apoptosis (programmed cell death, PCD) has been recognized as a possible outcome of DNA damage in 1980[232]. Our understanding on the mechanism derives from the investigation of apoptosis that occurs during the development of the nematode *Caenorhabditis elegans,* in which 131 cells die invariantly between worms at particular points during the development process[233]. Programmed cell death occurs during development, aging, as a homeostatic mechanism to maintain cell populations in tissues, or when cells are damaged. Cancer chemotherapy or irradiation result in DNA damage that can lead to apoptotic death through a p53-dependent pathway.

Inappropriate apoptosis contributes to many human conditions including neurodegenerative diseases, autoimmune disorders and many types of cancers.

Apoptosis is a coordinated and often energy-dependent process that involves a complex cascade of events. Two main apoptotic pathways have been discovered so far: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 1.14). They have been also shown to be linked and influence each other, and they both converge at the execution phase where caspases activate cytoplasmic endonucleases that degrade nuclear material and proteases that degrade cytoskeletal and nuclear proteins. Caspases (cysteinyl aspartic acid protease) are conserved cysteine proteases that are widely expressed in an inactive proenzyme form that require proteolytic cleavage for activation. Functionally, they can be divided into two groups: the initiator caspases, such as caspase 8 and 9 and the effector caspases, such as caspase 3 and 7.

An additional pathway has been described that involves perforin-granzyme-dependent cell death and T-cell mediated cytotoxicity[234] (Figure 1.14).

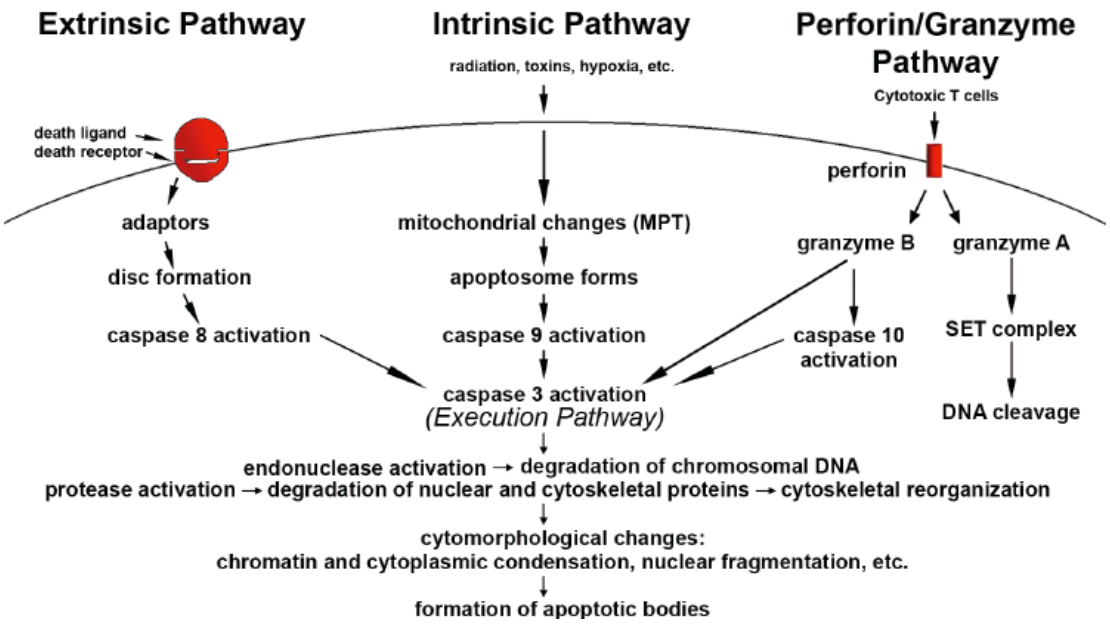


Figure 1.14: Apoptotic pathways

The two main pathways are the extrinsic and intrinsic apoptotic pathways as well as a perforin/granzyme pathway. Each is triggered by specific signals that result in the activation of their own initiator caspase, which in turn activates caspase-3. The perforin/granzyme pathway also works in a caspase-independent manner. In the final steps of apoptosis, characteristic cytomorphological changes can be observed such as chromatin condensation, formation of apoptotic bodies etc. Adapted from:[234]

The extrinsic pathway begins with the direct interaction of extracellular ligands with the death receptors located on the cell membrane of the cells destined for apoptosis. Ligand binding to death receptors such as TNF (tumor necrosis factor) receptors like Fas or CD95, and TRAIL (TNF related apoptosis inducing ligand) receptors result in the assembly and activation of the death receptor complex [235]. The complex consists of adapter proteins such as FADD (Fas-associated death domain) and the initiator caspase 8 that is activated through autoproteolytic cleavage [236]. Caspase 8 in turn activates caspase 3 and caspase 7, leading to subsequent apoptosis.

The tumor suppressor p53 has a crucial role in inducing the intrinsic pathway in response to genotoxic stress. p53 regulates the Bcl-2 family of proteins that govern the membrane permeability of the mitochondria. Bcl-2 proteins can be either pro-apoptotic (Bax, Bak, etc.) or anti-apoptotic (Bcl-2, Bcl-x, etc.). Once the pro-apoptotic proteins are activated, cytochrome c is released from the mitochondria, binds to and activates Apaf-1, forming an "apoptosome"[237]. Apoptosome formation results in a multicomponent adaptor complex that serves as a catalyst for caspase-9 oligomerization and mediates its auto-activation. The activated caspase 9 interacts with and activates caspase 3 [238]. Smac (small mitochondria-derived activator of caspases) is also released from the mitochondria, and it has been shown to suppress the action of IAPs (inhibitors of apoptosis proteins). IAPs (such as XIAP and survivin) are conserved proteins, which bind to both initiator and effector caspases and inhibit their proteolytic activity by covering the active site of the caspases and blocking the substrate entry [239, 240].

Interestingly, another cell death-inducing platform, termed 'Ripoptosome' was discovered recently. It assembles in response to genotoxic stress-induced depletion of IAPs, and forms independently of the extrinsic and intrinsic apoptotic pathways. It can stimulate both caspase-8-mediated apoptosis and caspase-independent necrosis[241].

The activation of the execution caspases results in cleavage of various substrates, including PARP, cytokeratins, and the endonuclease CAD. CAD is also released from the mitochondria in a later phase of apoptosis, along with AIF and endonuclease G that cause DNA fragmentation. Caspase-3 induces cytoskeletal reorganization as well.

The last stage of apoptosis is the phagocytic uptake of the cells in response to the externalization of phosphatidylserine on the surface of apoptotic cells [234].

1.9.3. Regulation of apoptosis by ubiquitination

The ubiquitin pathway regulates every phase of the programmed cell death through mono-or polyubiquitination of important factors. Ubiquitin expression has been found to be upregulated during apoptosis in the intersegmental muscle of insects and mature lymphocytes[242]; while the 26S proteasome controls the degradation of pro-and anti-apoptotic proteins. The Bcl-2 family of proteins are known targets for the UPS; degradation of pro-apoptotic factors such ad Bax and Bak has been shown to promote survival, while elimination of Bcl-2 and Bcl-x by the proteasome is required for apoptotic progression[242].

Caspase activity needs to be tightly regulated by subcellular localization, protein synthesis or ubiquitination. The IAP proteins possess RING domains and have been identified as ubiquitin E3 ligases for the effector caspases. In mammals, caspase 3 and 7 are monoubiquitinated through c-IAP (cellular IAP), which results in allosteric conformational impairment of the catalytic pocket of the caspase and steric interference with substrate entry. The *Drosophila* DIAP1 has been also shown to directly bind and ubiquitinate both the initiator caspase DRONC and the effector caspases drICE and DCP-1. Moreover, DIAP1 levels are also regulated by auto-ubiquitination that has been reported to be assisted by the SCF ligase complex[243]**.**

Another RING E3 ligase, Mdm2 is involved in the regulation of apoptosis through the suppression of p53. Under non-apoptotic conditions, p53 is ubiquitinated by Mdm2, exported to the cytosol and degraded by the UPS[244]. However, under pro-apoptotic circumstances, p53 ubiquitination is rapidly recovered by the action of the deubiquitinating enzyme HAUSP. In stress conditions, Mdm2 autoubiquitination and degradation is favored. Furthermore, $p14^{ARF}$, an alternate reading frame protein expressed from the INK4a locus, interacts with Mdm2, which leads to the relocalization

of Mdm2 from the nucleoplasm to the nucleolus, blocking the nuclear export of p53 by Mdm2 that is required for the degradation of the tumor suppressor protein[245].

Recently, several new studies emerged that link NEDD8 to apoptosis. MLN4924, the NEDD8 E1 inhibitor has been shown to induce apoptosis in several types of cancer, including liver cancer and head and neck cancers[246, 247]. In addition, the *Drosophila* inhibitor of apoptosis protein DIAP1 has been identified as a NEDD8 E3 ligase that NEDDylates itself, however, the functional consequences of the self-NEDDylation have not been determined yet[104].

1.10. Aims

My PhD project was aiming to further investigate the role of NEDD8 in diverse stress responses. The characterization of the ubiquitin-like modifier under proteasome inhibition and the utilization of the NEDD8 E1 inhibitor MLN4924 led us to the unexpected finding that the ubiquitin activating enzyme Ube1 activates endogenous NEDD8 *in vivo*. We characterized this phenomenon extensively, and showed that NEDD8 responds to heat shock and oxidative stress through the ubiquitin conjugation pathway.

We also investigated the NEDD8 chain formation upon MG132 treatment. One of our goals was to identify NEDD8 and NEDD8/ubiquitin mixed chains unambiguously by mass spectrometry. We achieved it by applying two different approaches. We have also shown that NEDD8 is present in the chains that are recognized by ubiquitin-binding domains.

In a parallel project, we aimed to characterize the role of the deNEDDylating enzyme NEDP1 in response to DNA damage. The NEDP1 ortholog in *C. elegans*, Ulp-3 has been previously investigated in collaboration with Anton Gartner's group and the enzyme has been found to be required for DNA damage-induced apoptosis in the worm germ line. We investigated whether the role of NEDP1 is conserved in human cell lines, and assessed its mechanism of action in the DNA damage-induced apoptosis pathway.

Chapter 2: Materials and Methods

2.1. Chemicals and suppliers:

Commonly used chemicals were of analytical grade, supplied by VWR and Sigma Aldrich. MG132 was purchased from Calbiochem, lactacystin from Enzo Life Sciences, MLN4924 from Millenium Pharmaceuticals, doxorubicin from Enzo Life Sciences, and actinomycin D and staurosporine were from Sigma. Lysyl endopeptidase C was purchased from Wako Chemical, and trypsin from Promega.

To investigate the effects of DNA damage induced apoptosis, cells were treated with 2-10 Gy of gamma irradiation using a Cs137 (IBL 437C, CIS bio international).

Centrifugation was done with a Heraeus Biofuge Pico Centrifuge with #3328 rotor.

2.2. Tissue culture and cellular assays

2.2.1. Cell lines and growth conditions

MCF7 human breast adenocarcinoma cells, U2OS human osteosarcoma cells, HeLa cervical cancer cells, HEK 293 human embryonic kidney cells and HCT116 colorectal carcinoma cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 50 U/ml Penicillin, 50 µg/ml Streptomycin and 10 % FCS (fetal calf serum) at 37°C, 5 % CO_2.

H1299 human non-small cell lung carcinoma cells were cultured in RPMI-1640 medium (Gibco) with 2 mM L-glutamine, 50 U/ml Penicillin, 50 µg/ml Streptomycin and 10 % FCS (fetal calf serum) at 37°C, 5 % CO2.

~90 % confluent cells were treated with trypsin-EDTA and replated to the desired confluency. Cells were used until they reached passage ~25.

PBS (phosphate buffered saline, Gibco) was used to wash the cells.

2.2.2. Generation of stable cell lines

For stable cell lines expressing His-NEDD8, a lentivirus vector generated by Liu Geng was used. HCT116 or U2OS cells were seeded in a 6-well plate. 80 µl of the lentiviral supernatant was added to the cells in the presence of 4 µg/ml polybrene. Medium was replaced with DMEM with 10 % FCS the next day. 2-3 days later 5 µg/ml puromycin was added to the medium. The stable cell lines were maintained in DMEM medium with 5 µg/ml of puromycin and the expression of His_6-NEDD8 was verified.

2.2.3. Long-term storage of cells

To prepare stocks for long-term storage, cells were grown to 90 % confluency, detached by trypsin, washed with PBS, and transferred to a 15 ml falcon tube. Cells were pelleted at 1000 rpm for 2 min in a Beckman centrifuge. The pellet was resuspended in FBS+ 10 % DMSO, and transferred to cryotubes (3 vials/ 75cm flask). The cells were first frozen at -80°C, before transferring them to liquid nitrogen storage. To recover cell lines, a vial was thawed in a 37°C water bath, and immediately plated in a flask with the adequate medium. Once cells settled, medium was changed to remove the DMSO.

2.2.4. Transfections

2.2.4.1. Cell plating

24 hrs before transfection, cells were washed twice with PBS, trypsinized and resuspended with RPMI or DMEM with 10 % FCS and antibiotics. Cell number was counted using a Hemocytometer.

2.2.4.2. Plasmid transfection with calcium-phosphate method

H1299 cells were transfected using the calcium-phosphate method. 1 hr prior transfection, RPMI media was replaced with DMEM to increase transfection efficiency. This is done as RPMI has high salt concentrations and interferes with the calcium-phosphate method. DNA solution (up to 20 µg of DNA in 437.5 µl ddH_2O) was mixed with 62.5 µl 2 M $CaCl_2$ in a tube. 500 µl 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES, pH 7.05) was added on a well of a 6 well plate. The DNA solution was added on the HBS dropwise and DNA/$Ca_3(PO_4)_2$ precipitates were monitored under microscopy before pipetted on the cells. 24 hrs after transfection media was replaced to RPMI. Cells were normally harvested 48 hrs after transfection.

2.2.4.3. Fugene transfection

All cell lines except H1299 were transfected with plasmid DNA using the Fugene HD Transfection Reagent (Roche) in 3:2 ratio of Fugene: DNA (µg). Cells were seeded on 6 well plates to reach 80-90 % confluency by the time of transfection. DNA was diluted in Opti-MEM serum-free medium. Fugene HD transfection reagent was added to the tube containing the DNA, vortexed and incubated for 15 min at RT. After incubation, the solution was added on the cells and incubated for 24-48 hrs prior harvesting.

2.2.4.4. siRNA transfection

siRNAs were purchased form Dharmacon as ***ON-TARGETplus*** SMARTpools (each pool contained 4 individual siRNAs targeting different parts of the gene of interest). Non-targeting siRNA from Dharmacon was used as a control. Prior to transfection, cells were seeded in 6-well plates with 2 ml DMEM and incubated overnight. 3.3 µl Lipofectamine RNAiMAX (Invitrogen) was diluted in 200 µl Optimem (Gibco), and

this was added to 0.5 µl of 20 µM siRNA in 200 µl Optimem, and then incubated for 20 mins at room temperature. The cells were harvested 48-72 hrs after the transfection. For the experiments with NEDP1 siRNA, 24 hrs after the transfection, the wells of the cells were trypsinized and split into 2 wells. The siRNA transfection was repeated the next day, and cells were harvested 4-5 days after the first transfection.

2.2.5. Ni^{2+}-NTA pulldown

Cells were grown on 10 cm culture dishes. Before harvesting, cells were washed twice with 3 ml ice cold PBS, and then scraped into 1 ml PBS. 200 µl of the sample was pelleted at 13000 rpm for 1 min and lysed in 200 ml 2X SDS (5 % m/v sodium dodecyl sulphate, 25 % glycerol, 150 mM Tris-HCl pH 6.8, 0.01 % m/v bromophenol blue). Lysates were passed through a 21 G needle 20 times and were boiled for 5 mins. 800 µl of the sample was lysed in 6 ml of 6 M GuaCl (6 M Guanidinium-HCl, 10 mM Tris-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0) with 10 mM imidazole and 10 mM β-mercaptoethanol. 50 µl of Ni^{2+}-NTA-agarose beads were added to the lysates and incubated for 4 hrs at room temperature or overnight in cold room. Beads were pelleted at 1500 rpm for 5 mins and were washed once with 750 µl 6 M GuaCl with 10 mM imidazole and 10 mM β-mercaptoethanol; four times with 750 µl 8 M Urea pH 6.3 (8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl) with 10 mM imidazole and 10 mM β-mercaptoethanol and 0.1 % Triton X-100. After each wash, beads were spun down at 7500 rpm for 1-2 min and the washing buffer was removed. 100 µl of 250 mM imidazole in 8 M Urea (pH 6.3) was added, and samples were incubated for 30 mins at RT, then spun at 13000 rpm for 10 min. The supernatant was collected, the eluate was mixed with 2X SDS and analysed by SDS-PAGE. His_6-NEDDylated proteins for mass spectrometry were isolated as described above, except that samples were eluted in 8 M Urea (pH 8.0) containing 200 mM imidazole. The second elution was digested in

solution with Lys-C for 12 hrs at room temperature. TAP-NEDD8 purification and proteomics analysis was done as described in[106].

2.2.6. Immunoprecipitation

U2OS or HEK 293 cells were cultured in 10 cm plates and transfected with HA-Ube1 or pcDNA3 plasmid using Fugene HD Transfection Reagent (Promega). Cells were lysed in 1% SDS, 5 mM EDTA, 10 mM iodoacetamide, 15 U/ml DNase I (Sigma) and protease inhibitors (Roche). After incubation at 95^{o}C for 5 min, the lysate was diluted 10 fold with non-denaturing lysis buffer (20 mM Tris pH 8, 150 mM NaCl, 1 % NP-40, 2 mM EDTA, protease inhibitors), and passed through a syringe. Anti-HA antibody coupled to sepharose beads (Sigma) was used for immunoprecipitation. Proteins were eluted with pH 2.8 elution buffer (Thermo). Eluates were split and in one set DTT (200 mM) was added. Samples were mixed with 8 M Urea and 2X SDS loading buffer and analysed by western blotting.

2.2.7. RNA isolation and Quantitative PCR (qPCR)

For detection of gene expression, RNA from the cells from 6-well plates was isolated using the Promega SV Total RNA Isolation system. cDNA was made using the Invitrogen SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR with 300 ng of RNA. The cDNA was diluted with H_2O (1:20 dilution) and 2 µl were used in 12 µl qPCR reaction with appropriate primers and TaqMan Gene Expression Master Mix (in case of the NEDD8 and NEDP1 detection) or SYBR Green PCR Master Mix (Applied Biosystems). Experiments were performed at least 3 times and data represent mean values +/- standard deviation.

2.2.8. Chromatin Immunoprecipitation (ChIP) and Sequential ChIP Assay

Cells were grown on 10 cm dishes. After harvesting, cross-linking was performed by incubating cells with 1 % formaldehyde for 10 min at room temperature. A final concentration of 125 mM glycine was added to terminate the reaction and incubated for 5 min at room temperature. Cells were washed twice with ice-cold PBS. Nuclei were isolated by incubation in cell lysis buffer (10 mM Tris-HCl pH 8.1, 10 mM NaCl, 0.2 % Nonidet P-40,) containing for 10 min on ice, followed by centrifugation for 2 min at 600 *g*. Nuclei were lysed in nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1 % SDS) containing Protease Inhibitor cocktail tablets (Roche) for 10 min at 4°C. The lysate was sonicated up to ~500 bp chromatin fragment and checked by agarose gel electrophoresis. After centrifugation at 16000 *g* for 10 min, soluble chromatin was diluted with immunoprecipitation (IP) dilution buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.01 % SDS, 1 % Triton X-100) at a 1:10 ratio of nuclei lysis buffer to IP dilution buffer. Chromatin was then pre-cleared by incubation with at least equal amount of preimmune serum or equal amount of control IgG for 2 hrs. Protein A was added and incubated for at least 2 hrs. Aliquots of pre-cleared samples (input) were saved. Remaining sample was incubated with 2.5 μg of anti-p53 (DO1) antibody per 1 ml IP reaction for 16 hrs at 4°C. Immune complexes were collected by incubation with pre-blocked Protein A Sepharose (Sigma) for 2 hrs at 4°C. Sepharose beads were washed twice with 0.5 ml of IP wash buffer 1 (20 mM Tris-HCl pH 8.1, 50 mM NaCl, 2 mM EDTA, 0.1 % SDS, and 1 % Triton X-100), once with IP wash buffer 2. Immune complexes were eluted twice with 150 μl of IP elution buffer (0.1 M $NaHCO_3$ and 1 % SDS). Cross-links were reversed by adding RNase A (3.3 g/ml) and NaCl (0.3 M) and incubating for 6 hrs at 65°C. Samples were digested with proteinase K (0.2 mg/ml) for

at least 2 hrs at 45°C. DNA was column purified by using ChIP DNA Clean and Concentrator Kit (Zymo Research).

Samples were analysed by qPCR. 2 µl of each eluate was used in a 12 µl reaction with appropriate primers. Primer sequences can be found in Table 2.1. Each experiment was performed at least 3 times and data represent mean values +/- standard deviation.

Real time PCR primers/ probes			
	Sense primer	Antisense primer	Probe
NEDD8	Obtained from Applied Biosystems as a validated mix of forward/reverse primers and probe		
Actin	CAACTCCATCATGAAGTGTGA	CAGGGCAGTGATCTCCTTCTG	-
NEDP1	Obtained from Applied Biosystems as a validated mix of forward/reverse primers and probe		
ChIP primers			
NEDP1 promoter	TGAACAGGAGGGCATGACAAG	CTTTTTGTTGCCTCTATCTTATTTTAACTG	
p21 promoter	GTGGCTCTGATTGGCTTTCTG	CTGAAAACAGGCAGCCC	
BAX promoter	TAATCCCAGCGCTTTGGAAG	TGCAGAGACCTGGATCTAGCAA	

Table 2.1. Primers used in gene expression and ChIP experiments

2.2.9. Caspase activity assays

A 5-days double treatment of NEDP1 or NT siRNA was performed in HCT116 cells. 6 hrs before harvesting, cells were either left untreated, or exposed to 7 Gy ionizing irradiation or 1 μM staurosporine. Caspase activity was measured using Caspase-Glo 3/7, 8 or 9 Assays (Promega), according to the manufacturers instructions. Luminescence signals were measured on luminometer (Berthold Microlumat Plus LB96V).

2.3. Bacteria and plasmid preparation

2.3.1. Media

LB (Luria-Bertani) media (1 % w/v bacto-tryptone, 0.5 % w/v bacto-yeast extract, 1 % w/v NaCl, pH 7.0), and LB Agar (LB +1.5 % bacto agar) plates with the appropriate antibiotic were prepared by the media services, MSI/WTB/JBC complex, University of Dundee and CRBM, Montpellier.

2.3.2. Preparation of chemically competent DH5α cells

5 ml LB medium was inoculated with a single colony of E.coli from a freshly streaked agar plate and incubated overnight at 37°C. This starter culture was used to inoculate 500 ml 2YT media. Once the culture reached OD_{600} =~ 0.6, it was chilled on ice for 5 min. The cells were collected by centrifugation at 2500 rpm for 10 min at 4°C. Bacterial pellet was then resuspended in 25 ml cold TSB (LB pH 6.1, 10 % w/v PEG, 5 % DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$). 2.5 ml sterile glycerol was added and the cells were incubated on ice for 10 min. The aliquots were snap-frozen in liquid nitrogen and stored at -80°C.

2.3.3. Transformation of chemically competent DH5α cells

5X KCM buffer (0.5 M KCl, 0.15 M $CaCl_2$, 0.25 M $MgCl_2$) was mixed with 1 μg of DNA and ddH_2O to a final volume of 100 ml at 1X KCM final. 100 ml E. coli DH5α competent cells were added, mixed and incubated first on ice for 20 min, followed by 10 min incubation at room temperature. 1 ml pre-warmed LB media was added to the cells and incubated for 1 hr at 37°C prior to plating on LB agar plates in the presence of appropriate selective antibiotic.

2.3.4. Plasmid DNA amplification and purification

5 ml LB with appropriate selective antibiotic (Amplicillin 100 mg/ml, Kanamycin 50 mg/ml) was inoculated with a single colony for small-scale plasmid purification. For "maxiprep", 250 ml LB media was inoculated overnight at 37°C with the 5 ml overnight culture. QIAprep Spin Miniprep Kit (Qiagen) and QIAGEN Plasmid Maxi Kit (Qiagen) were used for 5 ml and 250 ml culture respectively. The procedure was carried out according to the manufacturer's instructions.

2.3.5. Plasmid DNA quantification

DNA concentration was measured by spectrophotometer (eppendorf BioPhotometer) at OD_{260}. The purity of DNA was determined by the ratio of OD_{260} and OD_{280}.

2.3.6. DNA sequencing

DNA templates (20 ng/ml, min. volume 15 ml) and primers (3.2 pmol, min. volume 10 ml) were sent to The Sequencing Service, MSI/WTB/JBC complex, University of Dundee.

In the case of the NEDD8 mutants, sequencing was performed by Eurofins MWG with T7 primers.

2.3.7. Site-directed mutagenesis

Site-directed mutagenesis was performed with Phusion High-Fidelity polymerase (New England Biolabs). The reaction mixture was composed of 1X PCR buffer (NEB), 0.25 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, 0.5 U DNA polymerase 50 ng DNA template, 1 % DMSO. ddH_2O was added to make up the final volume to 50 ml. PCR was performed with the following cycling parameters: initial denaturation at 98°C for 30 sec; 18 cycles of subsequent denaturation at 98°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 2 min; final extension at 72°C for 7 min. Primers used for mutagenesis are shown in Table 2.2.

After the PCR was finished, 1 ml DpnI (20000 U/ml) was added to each PCR tube and incubated at 37°C for 2 hrs. DpnI only cleaves at methylated sites, hence it only digests the plasmid from bacteria but not from PCR products. 5 μl of the digested reaction was run on gel and compared to the undigested parental plasmid (they show different band pattern). 5 ml of each reaction was used for transformation and DNA isolated from miniprep was sequenced to confirm the mutation.

2.3.8. Agarose gel electrophoresis

DNA was resolved using 0.8-1 % w/v agarose gels (depending on the size of the DNA fragments of interest) in TAE buffer (40 mM Tris, 0.114 % glacial acetic acid, 1 mM EDTA pH 8.0) and 1X GelRed Nucleic Acid Stain (Biotium) (10,000X stock). Samples were mixed with 5X gel loading buffer (30 % Glycerol, 0.2 % w/v Orange G) and loaded on the gel, along with a 100 bp or 1 Kb DNA ladder (NEB). The gel was run at

60 V at room temperature and DNA was visualised using a transilluminator UV lightsource.

2.3.9. Plasmids

Eukaryotic expression plasmids used in this thesis can be found in Table 2.2.

Name	Template	PCR primers	Sources
HA-UBE1			Gift
His_6-NEDD8			Our lab
HA-NEDD8			Our lab
His_6-ubiquitin			Our lab
Mdm2			Our lab
Myc-Cullin4A			Our lab
NEDP1			Our lab
p53			Our lab
pcDNA3			Our lab
HA-NEDD8 L2A	HA-NEDD8	F:CGCTTCCCTTGGATCCGCCATTAAAGTGAAGACGC R:GCGTCTTCACTTTAATGGCGGATCCAAGGGAAGCG	
HA-NEDD8 K4A	HA-NEDD8	F:CCTTGGATCCCTAATTGCCGTGAAGACGCTGACC R:GGTCAGCGTCTTCACGGCAATTAGGGATCCAAGG	
HA-NEDD8 K6A	HA-NEDD8	F:GATCCCTAATTAAAGTGGCCACGCTGACCGGAAAGG R:CCTTTCCGGTCAGCGTGGCCACTTTAATTAGGGATC	
HA-NEDD8 T7A	HA-NEDD8	F:GATCCCTAATTAAAGTGAAGGCCCTGACCGGAAAGGAGATTG R:CAATCTCCTTTCCGGTCAGGGCCTTCACTTTAATTAGGGATC	
HA-NEDD8 K11A	HA-NEDD8	F:GAAGACGCTGACCGGAGCCGAGATTGAGATTGAC R:GTCAATCTCAATCTCGGCTCCGGTCAGCGTCTTC	
HA-NEDD8 E12A	HA-NEDD8	F:CGCTGACCGGAAAGGCCATTGAGATTGACATTG R:CAATGTCAATCTCAATGGCCTTTCCGGTCAGCG	
HA-NEDD8 I13A	HA-NEDD8	F:GCTGACCGGAAAGGAGGCCGAGATTGACATTGAAC R:GTTCAATGTCAATCTCGGCCTCCTTTCCGGTCAGC	
HA-NEDD8 D16A	HA-NEDD8	F:GAAAGGAGATTGAGATTGCCATTGAACCTACAGACAAG R:CTTGTCTGTAGGTTCAATGGCAATCTCAATCTCCTTTC	

HA-NEDD8 Q40A	HA-NEDD8	F:GAATCCCCCCACAAGCCCAGAGGCTCATCTAC R:GTAGATGAGCCTCTGGGCTTGTGGGGGGATTC	
HA-NEDD8 Y45A	HA-NEDD8	F:CACAACAGCAGAGGCTCATCGCCAGTGGCAAGCAGATGAATG R:CATTCATCTGCTTGCCACTGGCGATGAGCCTCTGCTGTTGTG	
HA-NEDD8 S46A	HA-NEDD8	F:GCAGAGGCTCATCTACGCCGGCAAGCAGATGAATG R:CATTCATCTGCTTGCCGGCGTAGATGAGCCTCTGC	
HA-NEDD8 K48A	HA-NEDD8	F:GGCTCATCTACAGTGGCGCCCAGATGAATGATGAGAAG R:CTTCTCATCATTCATCTGGGCGCCACTGTAGATGAGCC	
HA-NEDD8 D52A	HA-NEDD8	F:GCAAGCAGATGAATGCCGAGAAGACAGCAGC R:GCTGCTGTCTTCTCGGCATTCATCTGCTTGC	
HA-NEDD8 G64A	HA-NEDD8	F:GATTACAAGATTTTAGGTGCCTCAGTCCTTCACCTGGTG R:CACCAGGTGAAGGACTGAGGCACCTAAAATCTTGTAATC	
HA-NEDD8 V66A	HA-NEDD8	F:GATTTTAGGTGGTTCAGCCCTTCACCTGGTGTTGG R:CCAACACCAGGTGAAGGGCTGAACCACCTAAAATC	
HA-NEDD8 L69A	HA-NEDD8	F:GGTTCAGTCCTTCACGCCGTGTTGGCTCTGAG R:CTCAGAGCCAACACGGCGTGAAGGACTGAACC	
HA-NEDD8 K6R	HA-NEDD8	F:GATCCCTAATTAAAGTGCGTACGCTGACCGGAAAGG R:CCTTTCCGGTCAGCGTACGCACTTTAATTAGGGATC	
HA-NEDD8 K11R	HA-NEDD8	F:GACGCTGACCGGAAGGGAGATTGAGATTG R:CAATCTCAATCTCCCTTCCGGTCAGCGTC	

Table 2.2. Plasmid DNA information

For the plasmids constructed in the thesis, the template DNA with the PCR primers are shown. Plasmids were in pcDNA3 vectors, except HA-UBE1, which was in pCMV. This plasmid was a gift from Dr. Thimo Kurz. For other plasmids, sources are indicated. For PCR primers, both forward (F) and reverse (R) primers are 5'→3'.

2.4. Protein expression from bacteria

Construct name	Gene	Gene modification	Species	Vector	Tag	Cloning site
mRpn13 (1-407)-pGEX-4T1	Rpn13	wt	mouse	pGEX-4T1	GST	BamHI-XhoI
S5A (1-377)-pGEX-4T1	Rpn10	wt	human	pGEX-4T1	GST	XhoI-XhoI
hHR23A-UBA1-pGEX-4T1	Rad23	UBA1	human	pGEX-4T1	GST	BamHI-BamHI
hHR23A-UBA2-pGEX-4T1	Rad23	UBA2	human	pGEX-4T1	GST	BamHI-BamHI
hHR23B-UBA1-pGEX-4T1	Rad23	UBA1	human	pGEX-4T1	GST	BamHI-BamHI
hHR23B-UBA2-pGEX-4T1	Rad23	UBA2	human	pGEX-4T1	GST	BamHI-BamHI
hPLIC2 (UBA)-pGEX-4T1	PLIC2	UBA	human	pGEX-4T1	GST	BamHI-BamHI
pGEX-KG-UBA (Dsk2)	Dsk2	UBA	*S. cerevisiae*		GST	

Table 2.3. Constructs used for recombinant protein expression

All plasmids were constructed by and obtained from Dr. Koraljka Husnjak, except the Dsk2 UBA, which was a gift from Mark Laurence.

2.4.1. Transformation of chemically competent BL21 (DE3) Codon Plus cells

The list of bacterial expression vectors encoding the different UBA domains used in this thesis can be found in Table 2.3. Plasmids were transformed into 100 µl BL21-CodonPlus® Competent Cells and incubated on ice for 30 mins. The bacteria were heat shocked at 42°C water bath exactly for 2 mins, before being placed on ice for a further

minute. 1 ml prewarmed LB media was added and cells were incubated at 37°C before being plated on LB-ampicillin agar plates.

2.4.2. Protein expression

Single colony was picked to inoculate 5 ml LB with appropriate selective antibiotic (Amplicillin 100 mg/ml, Kanamycin 50 mg/ml) and incubated overnight at 37°C. This was further used to inoculate 200 ml LB media at 37°C, for 2-3hrs. Once OD_{600} reached 0.6-0.8, the culture was chilled on ice water for 10 min. Expression was induced with the addition of IPTG to a 100 μM final concentration and the culture was agitated at 20°C overnight.

2.4.3 Protein purification and coupling to Glutathione Sepharose beads

The overnight culture was pelleted at 5000 rpm for 20 min at 4°C, and the pellets re-suspended in 10 ml PBS with protease inhibitor (complete EDTA-free, Roche). 10 μl of 100 mg/ml lysozyme was added and incubated on ice for 30 min. Triton-X was added to 1 % final concentration and lysates were sonicated 3X 30s with 50 % amplitude on ice (Branson Digital Sonifier). Samples were centrifuged at 12,000g for 30 min at 4°C.

The supernatant was incubated with Glutathione Sepharose beads (GE Heatlhcare) overnight at 4°C. Beads were spun at 1500 rpm, 2 min at 4°C, washed three times with PBS and 0.1% Triton-X and once with PBS only. GST-tagged proteins coupled to beads were stored as a 50% slurry in PBS and 0.02% NaN_3 at 4°C.

2.5. Pulldown with GST-tagged UBA domains or UBA domain containing proteins

H1299 cells were grown in 15 cm dishes to ~90% confluency. Cells were either treated with the 30 μM MG132 for 4 hrs, or heat shocked at 43^{o}C incubator for 1 hour, or left untreated, as indicated. Before lysis, cells were washed twice with ice cold PBS, scraped off, and the pellet was resuspended in 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 10% glycerol, 10 mM EDTA, protease inhibitor and 50 mM iodoacetamide. The lysis was performed by syringing and incubation on ice. The lysate was cleared by centrifuging at 14,000 rpm, 10 min at 4 ^{o}C. 250 μl of the lysate per conditions was incubated with the different UBA domains overnight at 4 ^{o}C. Beads were washed 5 times with lysis buffer and proteins were eluted by boiling the samples in 60 μl 2X SDS. 20 μl was loaded on a 4-12% gel and analysed by western blotting with anti-ubiquitin and anti-NEDD8 antibodies. After western blotting, the membrane was stained with coommassie blue reagent to visualize the total proteins loaded.

2.6. Biochemical techniques

2.6.1. *In vitro* NEDD8 processing assay

For NEDD8 processing by NEDP1 or ULP-3, recombinant MBP-His-NEDD8-Ub fusion and the wild type and mutant enzymes were expressed and purified previously in the lab. The reaction was performed in 10 μl containing 600 ng of MBP-His-NEDD8-Ub, 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 200 ng NEDP1 or MBP-ULP-3 wt or MBP-ULP-3 Cys. Reactions were incubated for 10, 30 or 60 mins at 37°C and terminated by the addition of SDS loading buffer containing β-mercaptoethanol.

2.6.2 BCA protein assay

Protein concentration was measured by BCA Protein Assay (Thermo scientific). Reagent A and Reagent B was mixed in a 50:1 volume ratio and applied to dilute protein samples. Protein samples were incubated for 25 min in a 60°C water bath until the colour became light purple. The samples cooled down to room temperature and OD_{562} was measured by spectrophotometer. The protein concentration was determined from a standard curve using BSA.

2.6.3 SDS-polyacrylamide gel electrophoresis

Proteins were resolved with Invitrogen NuPage 12% or 4-12% Bis-Tris gels (Invitrogen) in Invitrogen Xcell SureLock Mini-Cell apparatus, which was filled with 500ml 1X MOPS running buffer (MOPS 50 mM, Tris base 50 mM, SDS 0.1%, EDTA 1 mM, pH 7.7). Alternatively, MES running buffer (MES pH 7.2 50 mM, Tris base 50 mM, SDS 0.1%, EDTA 1 mM, pH 7.3) was used to resolve low molecular weight proteins.

2.6.4 Coomassie staining

Polyacrylamide gel was incubated in Coomassie blue stain solution (0.1% w/v Coomassie Brilliant Blue, 40% v/v methanol, 7% v/v glacial acetic acid) followed by washes in Coomassie destain solution I (40% v/v methanol, 7% v/v glacial acetic acid) for 30min and three washes in Coomassie destain solution II (5% v/v methanol, 7% v/v glacial acetic acid) for several hours.

Coomassie staining of PVDF membrane was performed by incubation in Coomassie blue stain solution for 10 min, followed by several washes in ddH_2O.

2.6.5 Western immunoblotting

Proteins resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen) were transferred onto PVDF membrane (Millipore) using the Bio-Rad Mini Trans-Blot apparatus filled with 1L transfer buffer (0.2 M Glycine, 25 mM Tris, 20% methanol). Transfer was carried out at 25 mA overnight or at 70 V for 90 mins on ice.

Membranes were blocked by shaking with 5% milk solution (PBS with 5% skimmed milk and 0.1% Tween-20, boiled and filtered with dental napkins) for 1 hr at room temperature. Primary antibodies were diluted and stored in TBS 0.1% Tween-20 with 3 % BSA and 0.1% NaN_3 at 4°C and incubated with the membranes overnight at 4°C. Membranes were washed with 2X 10 mins PBS 0.1% Tween-20 prior to the incubation of the corresponding secondary antibodies (Sigma Aldrich), which were diluted 1:2000 in 5% milk solution, at room temperature for 1 hour. Membranes were washed with 4X 10 mins PBS 0.1% Tween-20 followed with 2X 5 mins PBS.

Detection was performed with ECL Western Blotting Detection Reagents (Amersham) prepared by mixing equal volumes of Reagent I and Reagent II. Membranes were incubated with the reagent solution mix for 1min, prior to being exposed to Medical Film (Konica Minolta) for an appropriate exposure time before being developed.

Name	Species	Stock Concentration	Dilution for WB	Sources
β-actin	Mouse		1:25000	Calbiochem
Cullin1	Mouse		0.5 μg/ml	In house
Cullin 4A	Rabbit	85 mg/ml	1:1000	Abcam
Cullin 3	Rabbit		1:1000	Gift
Flag	Mouse	3 mg/ml	1:3000	In house
HA	Mouse		1:3000	In house
His	Mouse		1:3000-5000	Clontech
Myc	Mouse	0.2 mg/ml	1:200	Santa Cruz
Mdm2 (4B2)	Mouse	2 mg/ml	1:2000	In house
NEDD8	Rabbit		1:1000	Epitomics
NEDP1	Sheep		1:1000	In house
Ubiquitin E1 Ube1	Rabbit	1 mg/ml	1:2000	Abcam
Uba6	Rabbit		1:2000	Gift
UbcH5	Sheep		1:20,000	In house
Cyclin E	Mouse			Gift
p53 (DO1)	Mouse	0.25 mg/ml	1:250-500	In house
p21	Mouse	0.2 mg/ml		Santa Cruz
SUMO-2/3	Sheep		1:2000	In house
α-Tubulin	Mouse		1:1000	Cell Signaling
Ubiquitin	Rabbit		1:2000	DAKO
Ubc12	Rabbit		1:2000	In house

Table 2.4. Primary antibodies used in the thesis.
WB: western blotting. Cullin 3 antibody was a gift from Matthias Peter, ETH Zurich.

2.7. Mass Spectrometric Analysis

The Lys-C-digested peptides were desalted on StageTips[248] and analyzed by LC-MS/MS on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany) coupled to a nanoflow HPLC (EASY-nLC system, Proxeon, Odense, Denmark). Only peptides with charge state equal or higher than 3 for the trypsin experiment, and 4 for the Lys-C experiment were fragmented by CID and recorded in the orbitrap analyser. Samples from the TAP-NEDD8 experiment were analysed on an LTQ-Orbitrap Classic instrument by Dr. Ivan Matic. Processing of raw MS data and the quantitative analysis were performed with the MaxQuant software[249, 250]. Mass additions of GG and LRGG were considered as variable modifications in "trypsin" experiments. Spectra were manually validated and assigned.

2.8. Handling of *S. pombe* and *C.elegans*

2.8.1 *Schizosaccharomyces pombe* strain, growth and media

Strain ID: FY7093, genotype: h+, ade6-M210 was used for the heat shock experiment. YES (YE+supplements) rich medium was prepared according to[251].

2.8.2 Heat shock and small scale protein extraction from *S. pombe*

Starter culture was inoculated from a freshly streaked plate and incubated for 1 day with agitation. A 150 ml YES medium was inoculated from the starter culture and incubated overnight, until OD_{600} has reached 0.3-0.6. This was split into 25 ml liquid cultures and incubated at 37-39°C for the indicated time periods. After centrifugation at 3000 rpm, 2

mins at 4°C, pellet was washed with ice-cold water and resuspended in lysis buffer (50 mM Tris pH7.5; 150 mM NaCl; 10 mM EDTA; 1% NP40; 10% glycerol, Roche protease inhibitor cocktail tablet and 50 mM iodoacetamide). After addition of 200 µl glass beads, cells were broken up in a FastPrep TM-24 beater (with a Hi Prep adapter 48 X 2 ml samples) beater for 3-times 40 sec, with 5 min rest in between. The tube was punctured with a heated needle, placed into a clean tube and spun at maximum speed for 10 sec. The insoluble pellet was removed by spinning for a further 5 min at max speed. Supernatant was mixed with equal amount of 2X SDS and boiled at 95°C for 5 min.

2.8.3 Heat shock and protein extraction from *C. elegans*

Bleached-synchronized L1 larvae stage worms were grown at 20°C on NGM agar plates seeded with *E. coli* HT115 strain. After reaching the L4 larvae stage, worms were subjected to a heat shock by transferring agar plates to 30°C for 5 hrs. Total protein was extracted by bead beating (Zirconia beads 0.7 mm, Biospect Products) in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA), clarified by centrifugation and boiled in SDS sample buffer. Protein were analysed by western Blot analysis.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad software. The two-tailed p values were determined using Student's t-test. Values $p<0.05$ were considered to represent statistically significant difference.

Chapter 3: NEDD8 responds to stress conditions via the ubiquitin pathway

3.1. Introduction

The ability of cells to adapt to environmental changes is essential for their survival. Diverse mechanisms have been evolved to protect cells from external or internal stress conditions. Upon stress stimuli, several cellular responses are coordinated by posttranslational modifications (PTMs), which alter the conformation and physicochemical properties of the target proteins. Regulation by PTMs provides a powerful and quick response to environmental changes, as the kinetics for PTMs control is faster than the regulation of protein expression levels.

The roles of ubiquitin and SUMO in stress responses have been extensively investigated. These modifications can alter protein properties such as stability, activity, localization and interaction with other proteins. One of the best-characterized and evolutionally conserved defence mechanisms is the heat shock response. In yeast, the ubiquitin gene is induced upon heat stress, enabling the organism to eliminate misfolded proteins via increased proteasomal degradation[252, 253]. Although higher eukaryotes have sophisticated repairing and refolding mechanisms [195], elimination of irreversibly damaged proteins through protein degradation is also part of the response [254]. Similarly, generation of reactive oxygen species can also cause protein damage and the proteasome is responsible for the removal of these damaged proteins. Furthermore, the activity of the proteasome itself is subject to oxidative inactivation that may result in the accumulation of ubiquitinated proteins [255]. SUMO conjugation is also induced upon cellular stresses, such as heat shock, osmotic stress and hibernation[256, 257]. Accumulation of SUMO conjugates upon proteasome inhibition have been previously noted[258], and a recent study provided insight into the role of SUMO in response to the accumulation of misfolded proteins[259]. The NEDD8 pathway has been well-characterized under homeostatic conditions (cullin-dependent

role), and more recently in ribosomal stress through regulation of ribosomal proteins [148]. However, the response of NEDDylation under broader environmental stress conditions remains unknown, especially with endogenously expressed proteins.

Historically ubiquitin-like molecules are thought to be activated and conjugated by their cognate enzymes. Recently examples of cross-activation have been emerged, unveiling another layer of regulation of ubls[102, 103]. Most of the ubl enzymes have striking affinity towards their cognate molecule, indicating that they must fulfill a rather specific role in the cells. The ubiquitin conjugating enzymes and E3 ligases however have been shown to regulate not only ubiquitin conjugation, but in certain circumstance other ubls too. The ubiquitin pathway appears to be a general, main regulator of many different cellular processes, while ubls are dedicated to a less broad range of functions.

3.2. Results

NEDD8 is 57 % identical and ~80 % similar to ubiquitin in sequence. The asterisks on the sequence alignment in Figure 3.1. show the amino acids that are identical in NEDD8 and ubiquitin. Moreover, their three-dimensional structure shares similarity as well (Figure 1.10), therefore it is likely that many ubiquitin-interacting proteins can also recognize NEDD8.

Figure 3.1: ClustalX aligment of human NEDD8 (accession number: NP_006147) and human ubiquitin (accession number: P62988)

An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties. A . (period) indicates conservation between groups of weakly similar properties. The residues are coloured according to their physicochemical properties (red: small + hydrophobic; blue: acidic; magenta: basic; green: hydroxyl + sulfhydryl + amine + G; grey: others).

3.2.1. Effect of the proteasome inhibitor MG132 on the NEDD8 signal

Previous studies using either overexpression of NEDD8 or cell lines stably expressing tagged NEDD8 showed that inhibition of the proteasome with MG132 results in increased NEDD8 conjugation to substrates[106]. We performed a shorter and longer time course experiment with the proteasome inhibitor, to investigate the phenomenon under endogenous conditions (Figure 3.2). MCF7 cells were treated with 30 μM MG132 for the indicated time intervals, directly lysed in 2X SDS and analysed by

western blotting. We found that the NEDD8 pathway responds to MG132 very rapidly (~5 min), and it is manifested mainly by an increase in high molecular weight conjugates. However, accumulation of NEDD8 conjugates does not lead to significant depletion of the free NEDD8 pool (see also data obtained with mass spectrometry (Figure 4.5) on the quantification of unconjugated NEDD8). We addressed the possibility that MG132 might affect NEDD8 at the transcriptional or translational level. Quantitative real time PCR using RNA from untreated or MG132 treated MCF7 cells shows no effect of MG132 on the levels of NEDD8 mRNA (Figure 3.3.a). Furthermore, inhibition of protein synthesis by cyclohexamide has no effect on the accumulation of NEDD8 conjugates upon MG132 treatment (Figure 3.3.b). Therefore, we concluded that the increase in NEDDylation by MG132 is a post-translational effect. It is also possible that NEDD8 itself may be a substrate for proteasomal degradation.

To detect NEDD8 signal in western blot analysis, we used a monoclonal NEDD8 antibody from Epitomics, which was initially developed by Millenium Pharmacheuticals. A synthetic peptide corresponding to residues in the N-term of human NEDD8 was used as immunogen. The antibody was also validated by Hjerpe et al. and showed no cross-reactivity with ubiquitin[260].

For the detection of ubiquitinated substrates, we used the polyclonal antibody from Dako, which has also been characterized by Hjerpe et al. and showed no cross-reactivity with NEDD8.

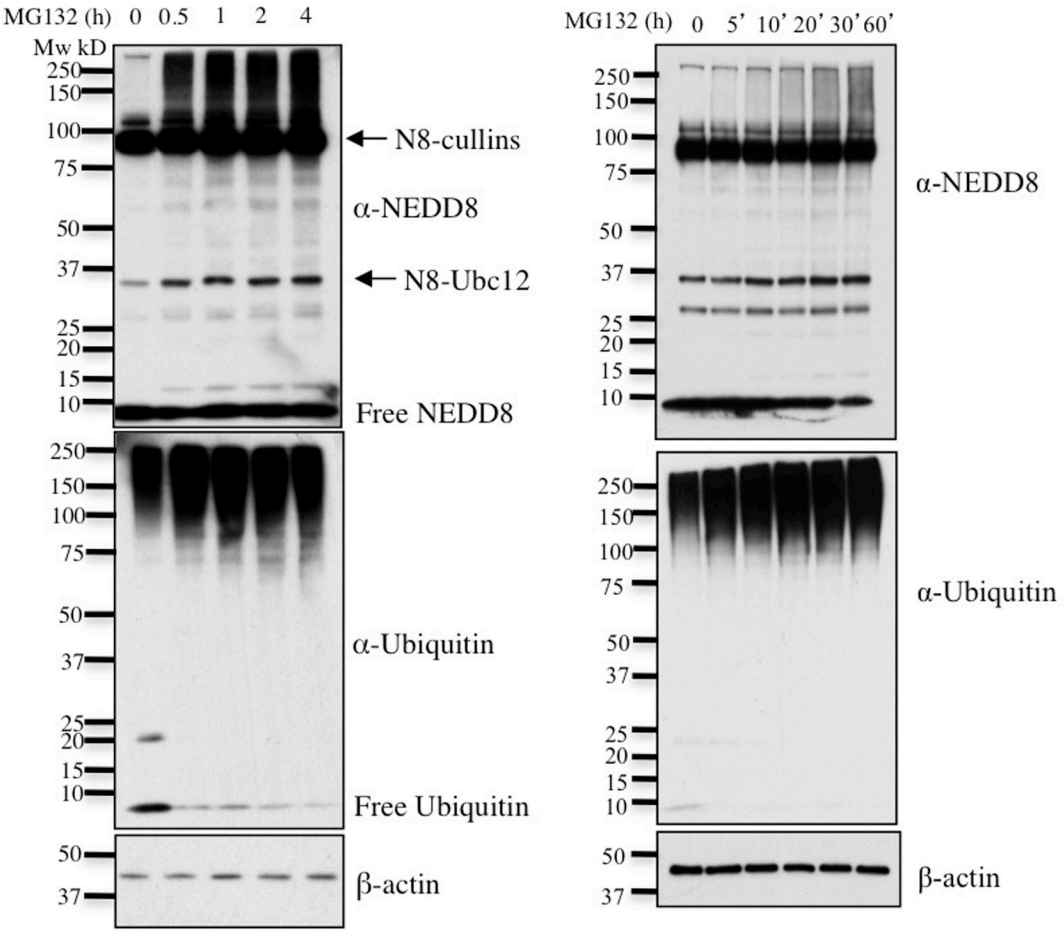


Figure 3.2: NEDD8 accumulates upon MG132 treatment.

MCF7 cells were treated with 30 μM MG132 for the indicated time intervals. Total cell extracts were analysed by western blotting with ubiquitin and NEDD8 antibodies.

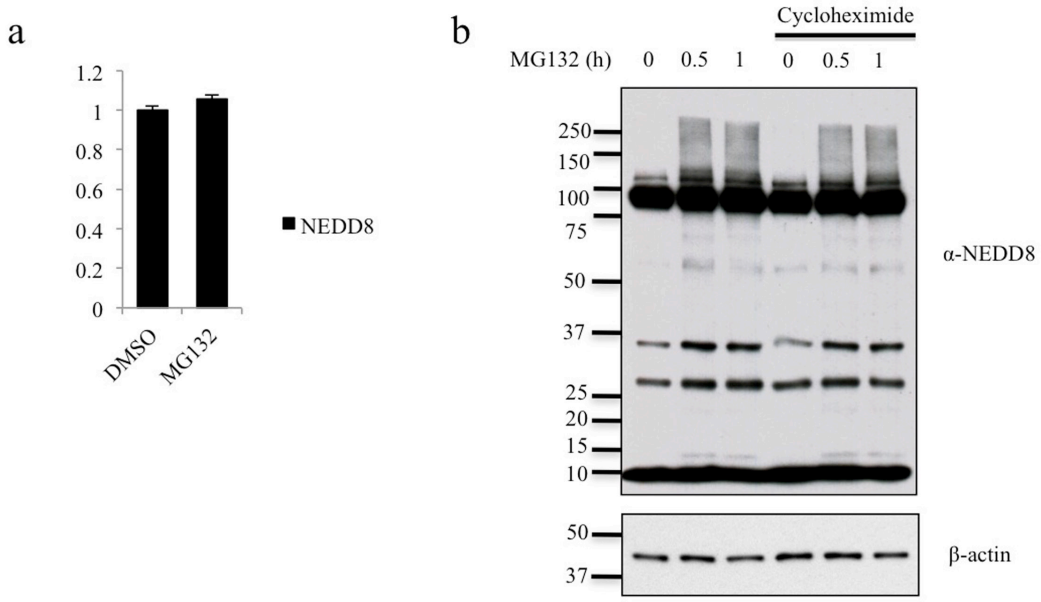


Figure 3.3: Increase in NEDDylation by MG132 does not depend either on transcription or translation.

(a) MCF7 cells were treated with 30 μM MG132 for 4 hrs. Quantitative real-time PCR for NEDD8 was carried out as described in Materials and Methods. The experiment was performed in triplicates; data are represented as mean +/-STDEV. **(b)** MCF7 cells were treated with 100 μM cycloheximide (CHX) just before treatment with 30 μM MG132 and harvested at the time points indicated. Total cell extracts were analysed by western blotting with NEDD8 antibody.

3.2.2. Increase in NEDD8 signal upon MG132 does not depend on the NEDD8 E1

Next, to investigate the specificity of the observed immunoreactivity and the regulation of NEDDylation we used either MLN4924, a specific inhibitor of NAE that blocks NEDDylation, or transfected cells with NEDD8 siRNAs [208, 209]. MCF7 cells were treated with non-targeting or NEDD8 siRNA for 48 hrs before harvesting. MLN4924 was applied in 1 μM concentration for 16 hrs. Under unstressed conditions, as expected, treatment with MLN4924 or knockdown of NEDD8 dramatically decreased NEDDylation in MCF7 cells (Figure 3.4.). However, under these experimental conditions, NEDDylation of cullins was not affected dramatically by NEDD8 knockdown. As cullins are the most efficient NEDD8 targets, we assume that the residual NEDD8 in cells is sufficient to support cullin NEDDylation[148]. The data also suggest that there is an excess of free unconjugated NEDD8 in cells. It is also possibly that there is a pool of NEDD8 in cells that can not be knocked down efficiently under the conditions used.

Treatment with 30 μM MG132 for 4 hrs causes a dramatic increase in NEDDylation. Surprisingly, inhibition of NAE by MLN4924, while it blocked cullin NEDDylation had no effect on the accumulation of additional NEDDylated species (Figure 3.4.). Under these conditions, knockdown of NEDD8 dramatically decreased the observed immunoreactivity, confirming the identity of these species as NEDD8 conjugates. The data suggest that upon proteasomal inhibition, NEDD8 conjugation is dramatically increased through an NAE independent pathway.

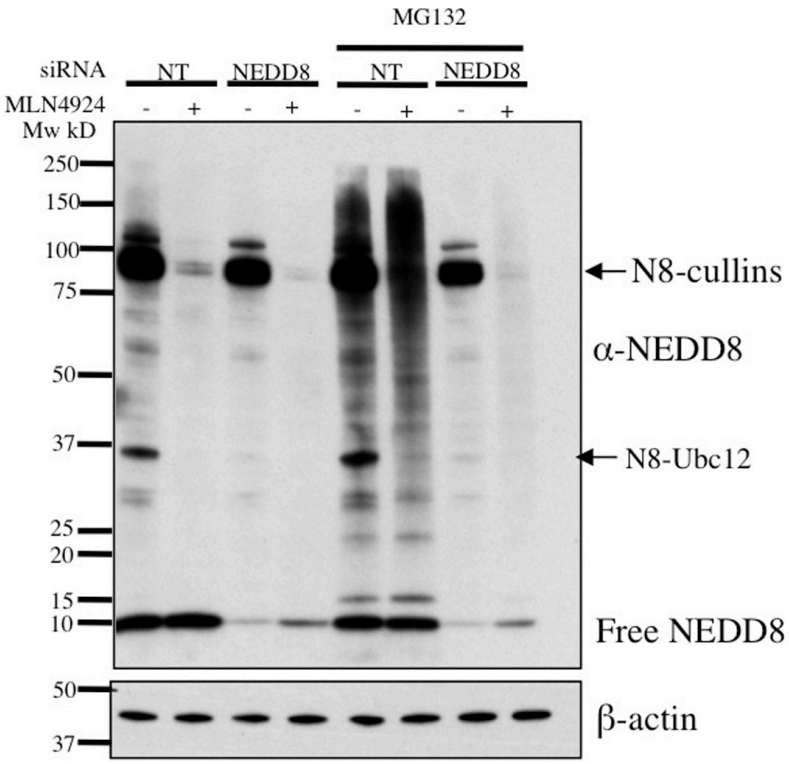


Figure 3.4: NAE-independent increase of NEDDylation.

MCF7 cells were transfected with non-target or NEDD8 siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. 4 hrs before harvesting, cells were treated with 30 μM MG132 where indicated. Total cell extracts were analysed by western blotting.

3.2.3. The ubiquitin E1 enzyme Ube1, but not Uba6 mediates increase in NEDD8 conjugates upon proteasome inhibition

The data suggest that NEDD8 modification of certain substrates could occur through an alternative conjugation pathway, independent of NAE activation. Since NEDD8 shares 57 % identity to ubiquitin, and previous *in vitro* studies showed that NEDD8 could be activated by the ubiquitin E1, Ube1[261], we tested whether the ubiquitin activating enzymes are responsible for mediating protein NEDDylation *in vivo*. We performed the

experiment described in Figure 3.4., this time transfecting MCF7 cells with siRNA against Ube1 or Uba6, the two known E1 ubiquitin activating enzymes[14]. Knockdown of Ube1 had no effect on the NEDD8 profile under homeostatic conditions, however, NEDD8 accumulation upon MG132 treatment was dramatically reduced upon Ube1 knockdown (Figure 3.5. a). To confirm the result, we performed the experiment in different cell lines. Figure 3.5. b shows the same experiment performed in HeLa cells. Similar effects were observed with individual siRNAs targeting different parts of the Ube1 mRNA sequence (Figure 3.6.). On the other hand, decrease in Uba6 levels did not have any effect on protein NEDDylation in any tested conditions, suggesting that this enzyme doesn't play role in NEDDylation (Figure 3.7.). Interesting to note that Ube1 siRNA did not have detectable effect on ubiquitin conjugates. It is possible that Ube1 is in excess in cells, and the remaining enzymes are still sufficient to activate ubiquitin, but not the 'poor' substrate, NEDD8. Detecting changes in high-molecular weight ubiquitin conjugates on western blot is difficult, since the signal is saturated, and the species the antibody detects under these conditions might be aggregates. In this case, our method would not be suitable to detect changes in ubiquitination upon Ube1 knockdown.

Next we aimed to confirm that the Ube1-mediated increase in NEDD8 conjugation is indeed triggered by proteasome inhibition and not due to other effects MG132 might have in cells. Therefore we used a more specific inhibitor of the proteasome, lactacystin (Figure 3.8.) [262]. We observed similar effects to MG132, although the NEDD8 response to MG132 treatment was stronger than to lactacystin. This suggests that MG132 probably has some off-target effects as well, but also shows that NEDD8 responds to a more specific proteasome inhibitor.

These results indicate that there is an increase in protein NEDDylation upon proteasome inhibition, which depends on Ube1, but probably not on Uba6.

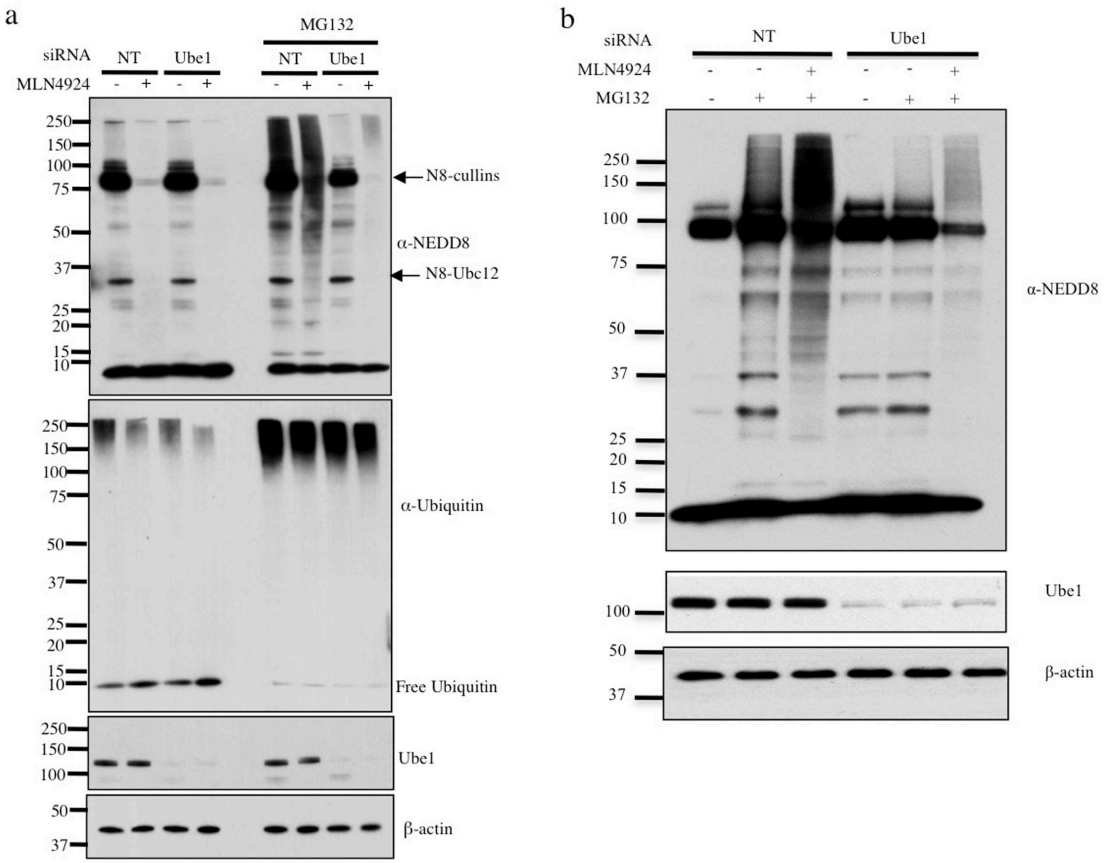


Figure 3.5: Ube1 activates NEDD8 *in vivo* upon proteasome inhibition.

(a) MCF7 cells were transfected with non-target or Ube1 siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. 4 hrs before harvesting, cells were treated with 30 μM MG132 where indicated. Total cell extracts were analysed by western blotting. (b) HeLa cells were transfected with non-target or Ube1 siRNAs for 48 hrs, then treated or untreated with 1 μM NAE inhibitor (MLN4924) for 16 hrs. 4 hrs before harvesting, cells were treated with 30 μM MG132 where indicated. Total cell extracts were analysed by western blotting.

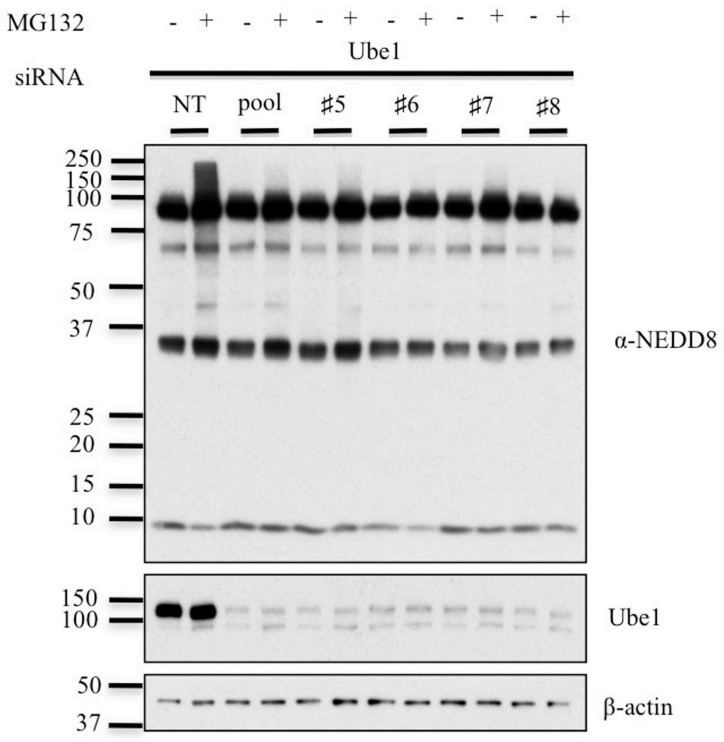


Figure 3.6: Test of individual Ube1 siRNAs

MCF7 cells were transfected with individual Ube1 siRNAs for 48 hrs. 4 hrs before harvesting, cells were treated with 30 μM MG132 where indicated. Total cell extracts were analysed by western blotting.

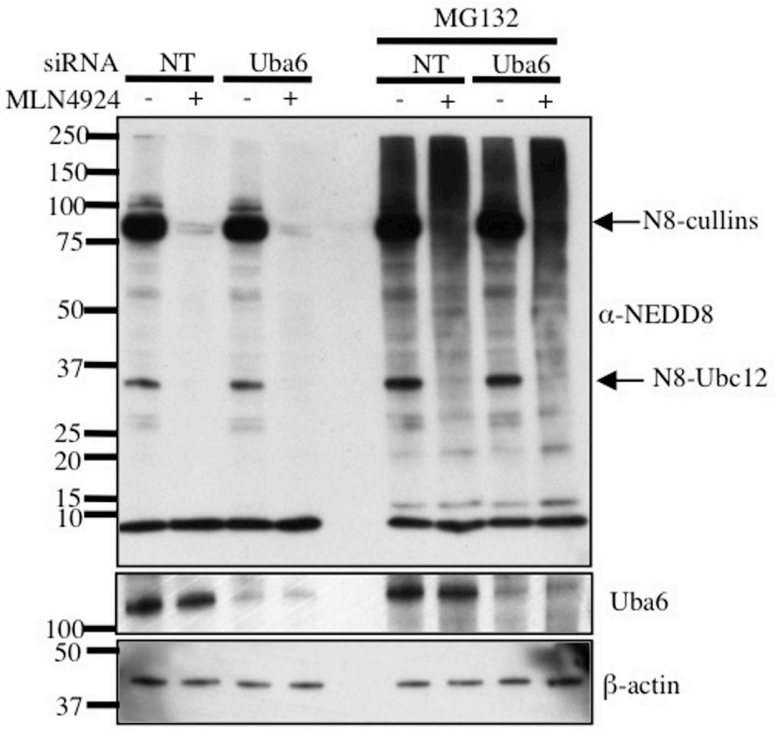


Figure 3.7: Uba6 knockdown experiment suggests that Uba6 does not play a role in NEDDylation.

Experiment performed as in Figure 3.5. with the exception of using Uba6 siRNA instead of Ube1.

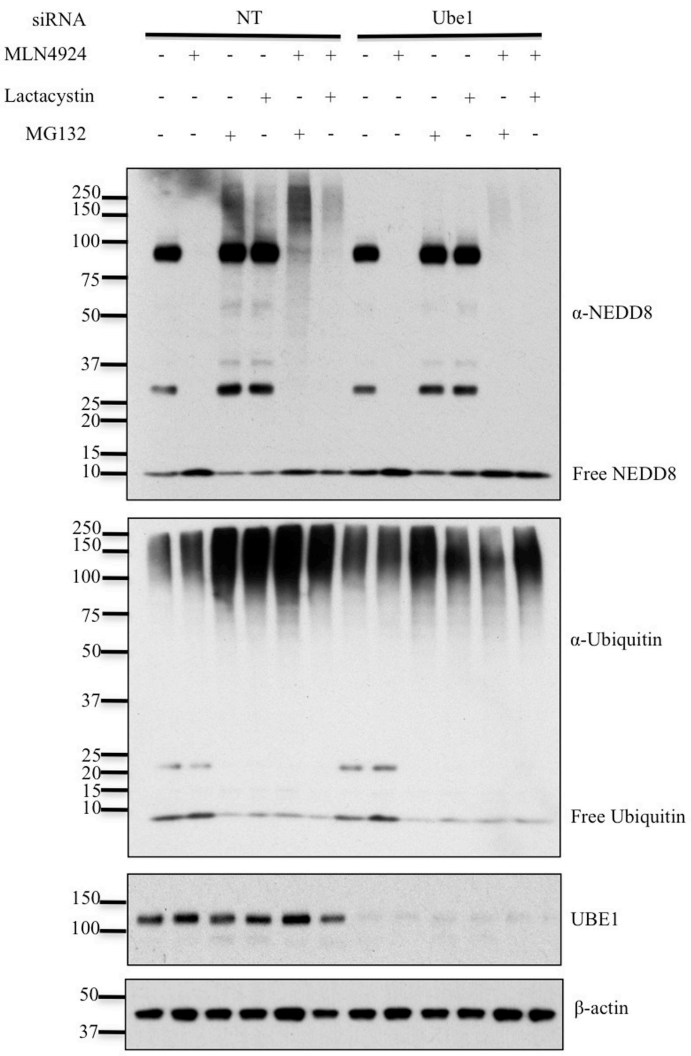


Figure 3.8: Lactacystin induces NEDD8 accumulation.

MCF7 cells were transfected with non-target or Ube1 siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. 4 hrs before harvesting, cells were treated either with 30 μM MG132 or 300 μM lactacystin where indicated. Total cell extracts were analysed by western blotting.

3.2.3. NEDD8 response to MG132 treatment upon knockdown of Ubc12, UbcH5a and Mdm2

We tested whether the best-characterised NEDD8 E2 Ubc12 and Mdm2, the dual ubiquitin/NEDD8 E3 ligase have an effect on the observed increase in NEDD8 conjugation upon MG132. Cells were treated with Mdm2 or Ubc12 siRNAs for 48 hrs before exposed to MG132 treatment for 4 hrs. Cells extracts were analysed by western blotting. The result suggests that Mdm2 and Ubc12 knockdown do not abolish increased NEDD8 conjugation (Figure 3.9).

We also tested whether the knockdown of a single ubiquitin E2 enzyme, UbcH5a would inhibit NEDD8 response to MG132. UbcH5a is a very diverse ubiquitin E2 that controls ubiquitination of many targets. However, in our experiment it did not affect the NEDD8 response to MG132 (Figure 3.10). Important to note that the knockdown of UbcH5a was not very efficient in this experiment. Additionally, there might be a specific subset of substrates the are NEDDylated in a UbcH5a-dependent manner, but their disappearance can not be visualised from total cell extract. In the paper of Hjerpe et al. the authors showed that NEDD8 can form a thiolester bond with several ubiquitin E2s in vitro[260], suggesting that multiple ubiquitin E2 conjugating enzymes are responsible for NEDD8 conjugation under stress.

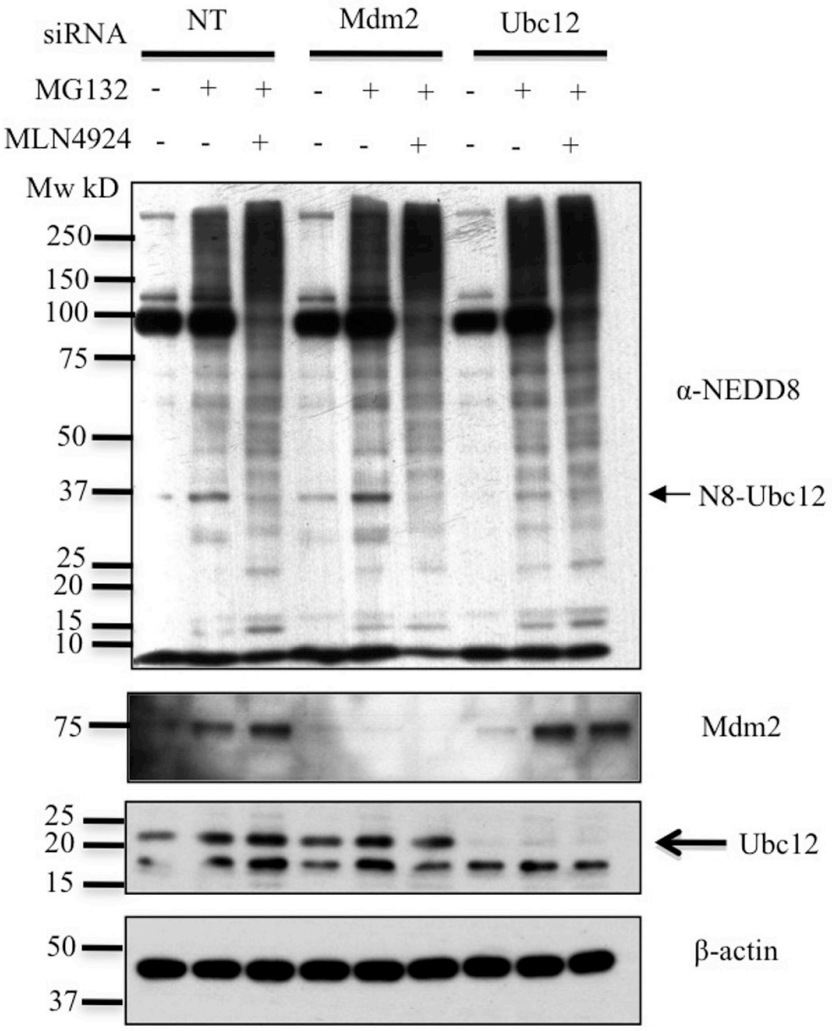


Figure 3.9: Effects of Mdm2 and Ubc12 knockdown on the NEDD8 response to MG132.

MCF7 cells were transfected with non-target, Mdm2 or Ubc12 siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. 4 hrs before harvesting, cells were treated or not with 30 μM MG132. Total cell extracts were analysed by western blotting.

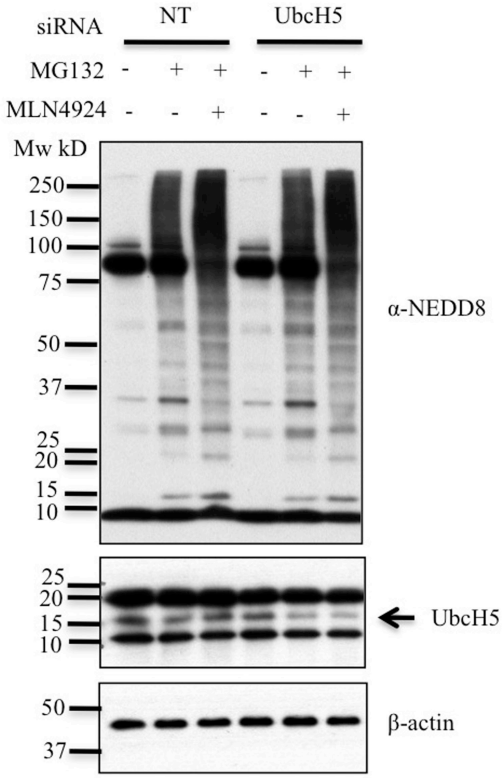


Figure 3.10: Effects of UbcH5a knockdown on the NEDD8 response to MG132.

MCF7 cells were transfected with non-target or UbcH5a siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. 4 hrs before harvesting, cells were treated or not with 30 μM MG132. Total cell extracts were analysed by western blotting.

3.2.4. NEDD8 responds to heat shock in a Ube1-dependent manner

Ubiquitin and ubiquitin-like molecules respond to a variety of environmental stresses as part of the cellular response. However, while the role of NEDDylation is well-documented under unstressed conditions, there is little knowledge upon stress conditions. Our data have revealed that upon proteasome inhibition NEDD8 conjugation is dramatically increased, unexpectedly through the ubiquitin E1 activating enzyme Ube1. We then determined whether this is an unique phenomenon, or NEDD8 responds

to other environmental stresses such as heat shock and oxidative stress in a similar manner. MCF7 cells were shifted from 37^{o}C to 43^{o}C for different intervals, and total cell extracts were analysed with a NEDD8 specific antibody. We found that NEDDylation rapidly increases in heat shocked cells (within 10'), compared to the unstressed cells (Figure 3.11). We also looked at the recovery period of heat shock: cells were shifted to 43^{o}C for 60' followed by incubation at 37^{o}C for 120' to recover. In this case, we observed a decrease (recovery) in NEDDylation, suggesting that the NEDD8 response to heat shock is a regulated process (Figure 3.11). Inhibition of NAE by MLN4924 had no effect on the NEDD8 response to heat shock, indicating that the increase in NEDDylated species is NEDD8 E1-independent (Figure 3.12). However, when cells were transfected with Ube1 siRNA, the NEDD8 response to high temperature was no longer observed (Figure 3.12). The data reveal a physiological stress condition where NEDD8 responds through Ube1.

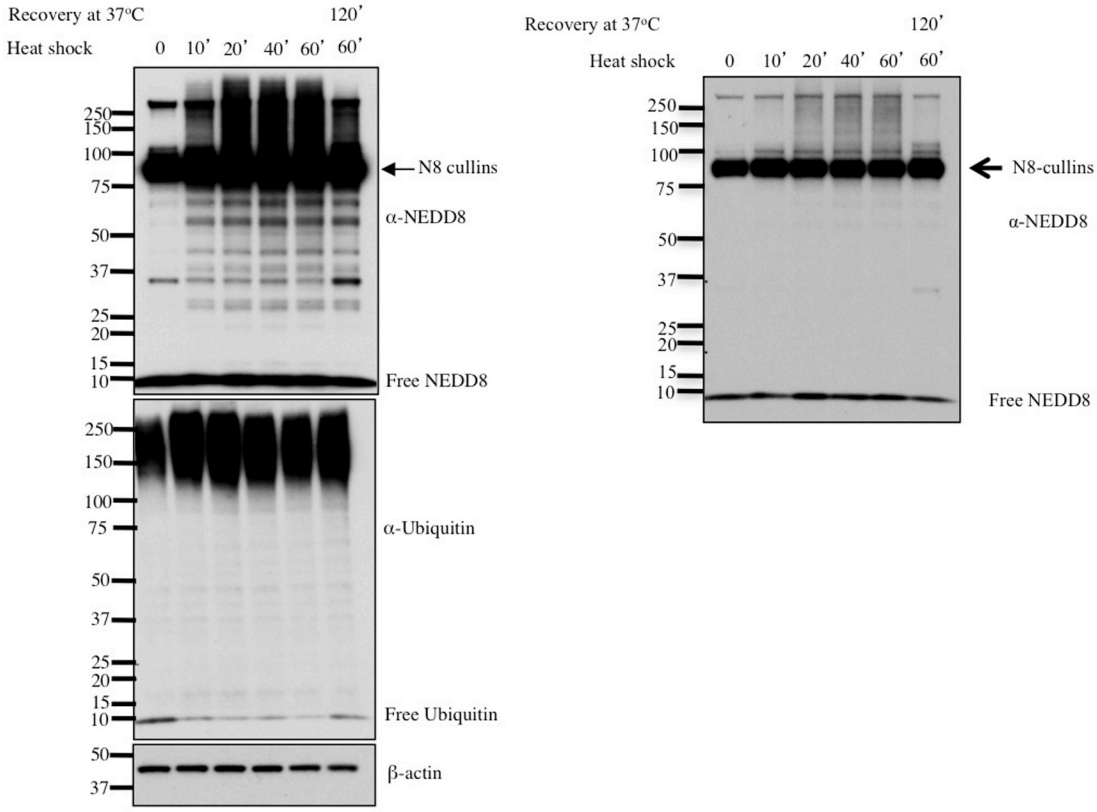


Figure 3.11: NEDD8 responds to heat shock.

MCF7 cells were grown under normal condition (37^{o}C). For the indicated periods of time, cells were shifted to 43^{o}C or left at 37^{o}C. After heat shock, cells were either immediately harvested, or let to recover at 37^{o}C for 2 hrs. Total cell extracts were immuno-blotted with the indicated antibodies. The two NEDD8 blots represent a longer and a shorter exposure of the same blot.

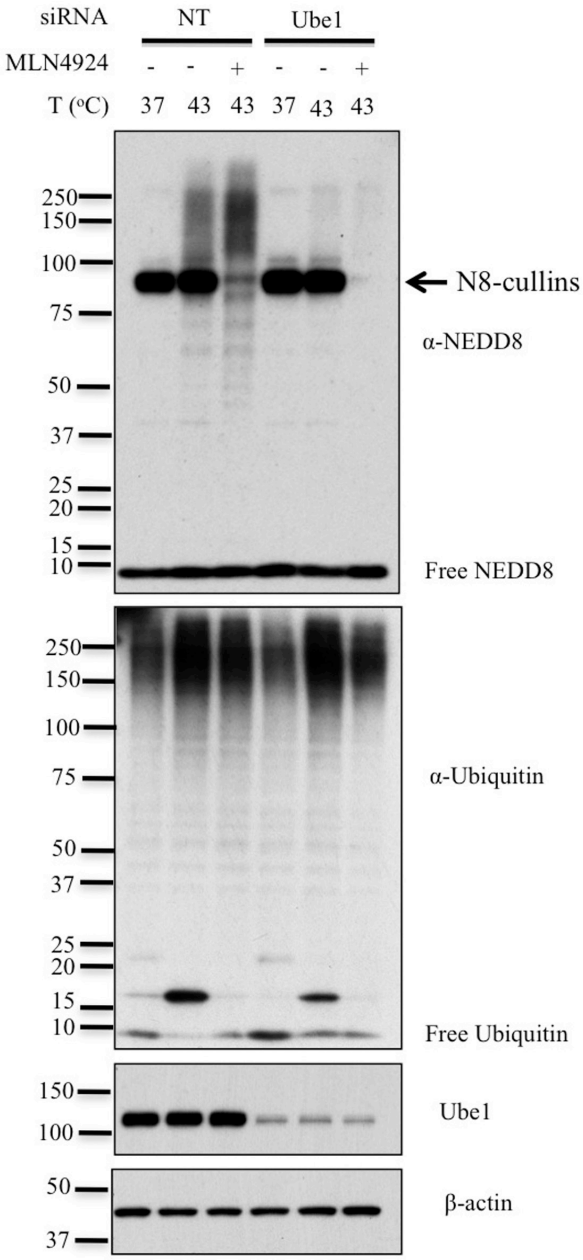


Figure 3.12: Heat shock triggers increased NEDD8 conjugation in an Ube1-dependent fashion.

MCF7 cells were transfected with non-target or Ube1 siRNAs for 48 hrs, then treated or untreated with 1 μM MLN4924 for 16 hrs. Before harvesting, cells were either left untreated (37°C) or exposed to heat shock (43°C). Total cell extracts were analysed by western blotting.

3.2.5. NEDD8 responds to oxidative stress

Another commonly studied cellular stress is the response to reactive oxygen species. Previous studies have shown that while ubiquitin conjugates are not severely affected by oxidative stress caused by H_2O_2, SUMO conjugation is reduced [200]. We found that H_2O_2 causes a dramatic increase in NEDD8 conjugation at concentrations (1 mM) where SUMO-2/3 conjugates are decreased (Figure 3.13). Similarly to proteasomal inhibition and heat shock, the observed increase in NEDD8 conjugates does not depend on NAE but rather on Ube1 (Figure 3.14). The above data demonstrate that NEDD8 responds to a variety of cellular stress conditions but surprisingly NEDD8 conjugation is mediated through the ubiquitin E1 activating enzyme Ube1.

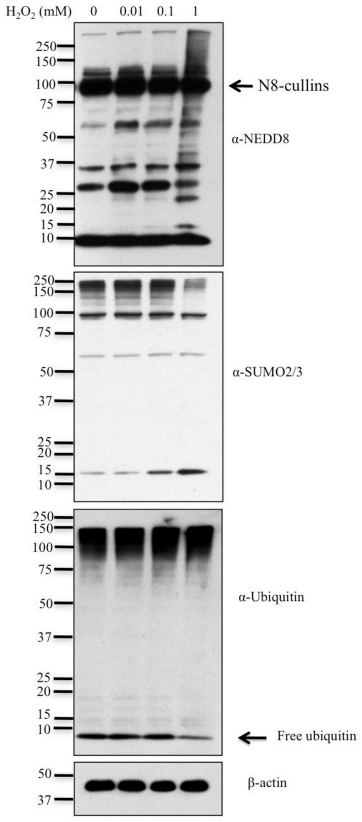


Figure 3.13: NEDD8 responds to H_2O_2 treatment.

MCF7 cells were treated with increasing doses of H_2O_2 for 1 hr and total cell lysates were analysed by western blotting.

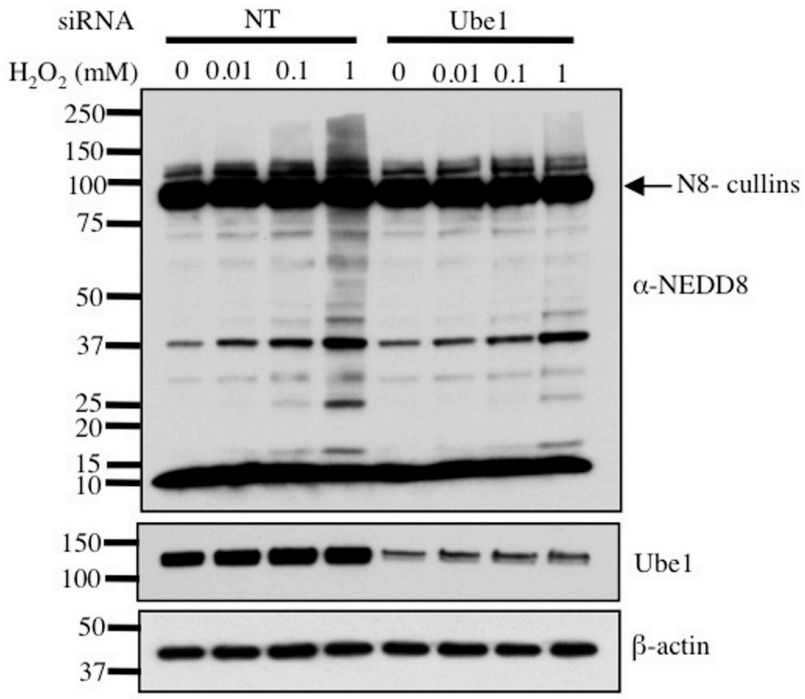


Figure 3.14: Oxidative stress results in increased NEDD8 conjugation in an Ube1-dependent fashion.

MCF7 cells were transfected with non-target or Ube1 siRNAs for 48 hrs, then exposed to H_2O_2 for 1 hr. Whole cell lysates were analysed by western blotting.

3.2.6. Ubiquitin depletion is sufficient to allow NEDD8 activation by the ubiquitin E1 activating enzyme Ube1

Early biochemical studies and as presented in a paper by Hjerpe et al., show that *in vitro*, Ube1 has a very low activity towards activating NEDD8 compared to ubiquitin[260, 261]. However *in vivo*, our data show that this activation occurs under defined cellular stress conditions. A common feature of the above characterised cellular insults is that they all result in depletion of free ubiquitin, therefore altering the ratio between the unconjugated pools of NEDD8 and ubiquitin that are available for the E1 enzymes. Studies have suggested that free ubiquitin in cells constitutes a cellular signal

and its depletion initiates a stress response [11, 263]. We therefore tested whether free ubiquitin depletion alone in the absence of any stress would allow NEDD8 to be conjugated through Ube1. MCF7 cells were transfected with siRNAs against ubiquitin for a relatively short period of time (15 hrs) so that there are no perturbations in cell growth. Knockdown of ubiquitin in the absence of any additional stress is sufficient to cause the increase of NEDD8 conjugates that depends on Ube1 (Figure 3.15). SUMO-2/3 conjugation that also increases upon proteasome inhibition and heat shock is not affected by ubiquitin knockdown (Figure 3.15). The data suggest that decrease in ubiquitin levels is at least part of the mechanism that allows NEDD8 conjugation through Ube1.

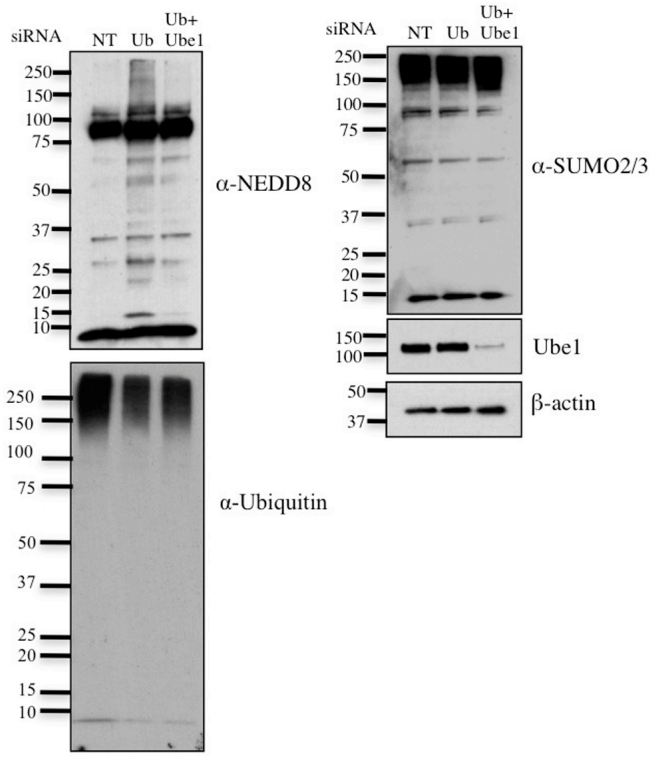


Figure 3.15: Decrease in free ubiquitin level triggers increased NEDD8 conjugation through Ube1.

MCF7 cells were transfected with non-target or Ube1 siRNAs for 48 hrs and ubiquitin (UBB) siRNA for 15 hrs, lysed and immunoblotted with the appropriate antibodies.

3.2.7. Ubiquitin overexpression does not prevent the NEDD8 response to MG132

Even though little work has been done to investigate the effects of free ubiquitin depletion, a paper from Hanna et al. has shown that ubiquitin overexpression provides resistance to stress conditions that are associated with free ubiquitin depletion[202]. If free ubiquitin depletion is the only event that is required for Ube1-mediated NEDD8 activation, we reasoned that overexpression of ubiquitin would rescue this phenotype. Different amounts of ubiquitin construct were transfected in MCF7 cells for 48 hrs before MG132 treatment and harvesting. Analysis of the total cell lysates by western blotting with NEDD8 antibody showed that NEDD8 responds to MG132 treatment, even when 2 μg of ubiquitin-coding plasmid was used (Figure 3.16). This result can be interpreted in different ways. It is possible that the ubiquitin system prefers to engage in NEDD8 conjugation under stress conditions, even when there is sufficient amount of ubiquitin available. If this is true, free ubiquitin depletion must be only part of the mechanism that triggers NEDD8 activation and the depletion of ubiquitin may create a stress response that would promote activation of NEDD8 by Ube1. Another explanation is that the exogenous ubiquitin is not used in a same way as the endogenous; hence it cannot rescue the phenotype.

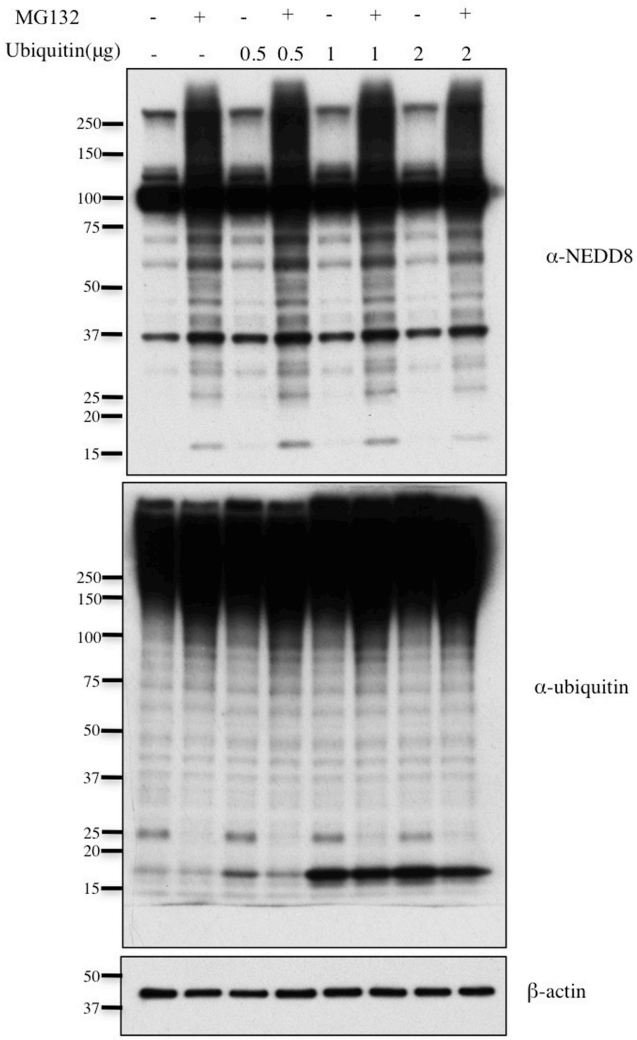


Figure 3.16: Ubiquitin overexpression does not prevent the NEDD8 response to MG132.

MCF7 cells were transfected with different amounts of plasmids encoding ubiquitin. 'Ubiquitin (µg)' indicates the amount of plasmid transfected. 48 hrs after transfection, cells left untreated or treated with 30 µM MG132. Total cell extracts were analysed by western blotting.

3.2.8. Ube1 forms a thiolester linkage with NEDD8 *in vivo*

We have shown that the NEDD8 response to stress conditions is mediated through Ube1. In order to prove that Ube1 is indeed able to activate NEDD8 and this is the

mechanism by which it promotes increased NEDD8 conjugation, we tested whether Ube1 forms a thiolester bond with NEDD8 in cells. HA-tagged Ube1 was overexpressed in U2OS cells. We performed an HA immunoprecipitation in conditions that allowed us to preserve the thiolester bonds (non-reducing conditions, addition of alkylating agent to the buffer). After the elution, samples were divided, and reducing agent, DTT was added to half of the samples. The samples were analysed by western blotting, with NEDD8 or ubiquitin antibodies that detected a band of the expected size, which was sensitive to reducing agents (Figure 3.17. a). In a similar experiment we knocked down ubiquitin in HEK 293 cells to test whether it affects the thiolester intermediate. Surprisingly, ubiquitin siRNA treatment did not result in an increased thiolester formation between Ube1 and NEDD8 (Figure 3.17. b). In the immunoprecipitation experiments, Ube1 formed a thiolester linkage with endogenous NEDD8 under normal conditions, and we saw no further increase with ubiquitin knockdown or other stress conditions. This is probably due to the overexpression of Ube1. If there is more Ube1 in cells, it becomes available for NEDD8 activation. This also suggests that the ratio between the free NEDD8, ubiquitin, and Ube1 determines which ubl is activated.

The western blot at the lowest panel of Figure 3.17.b shows the total NEDDylation. As mentioned above, overexpressed Ube1 forms a thiolester bond with endogenous NEDD8 under normal conditions. However, this alone does not result in a detectable increase in NEDD8 conjugation (second lane), only when it is triggered by stress (ubiquitin knockdown, third panel). This observation raises the possibility that changes in the ratio between free ubiquitin/NEDD8/available Ube1 is only part of the mechanism that allows NEDD8 engagement into the ubiquitin pathway. There might be a more specific regulation for the switch, possibly through a stress-induced posttranslational modification of NEDD8 or Ube1.

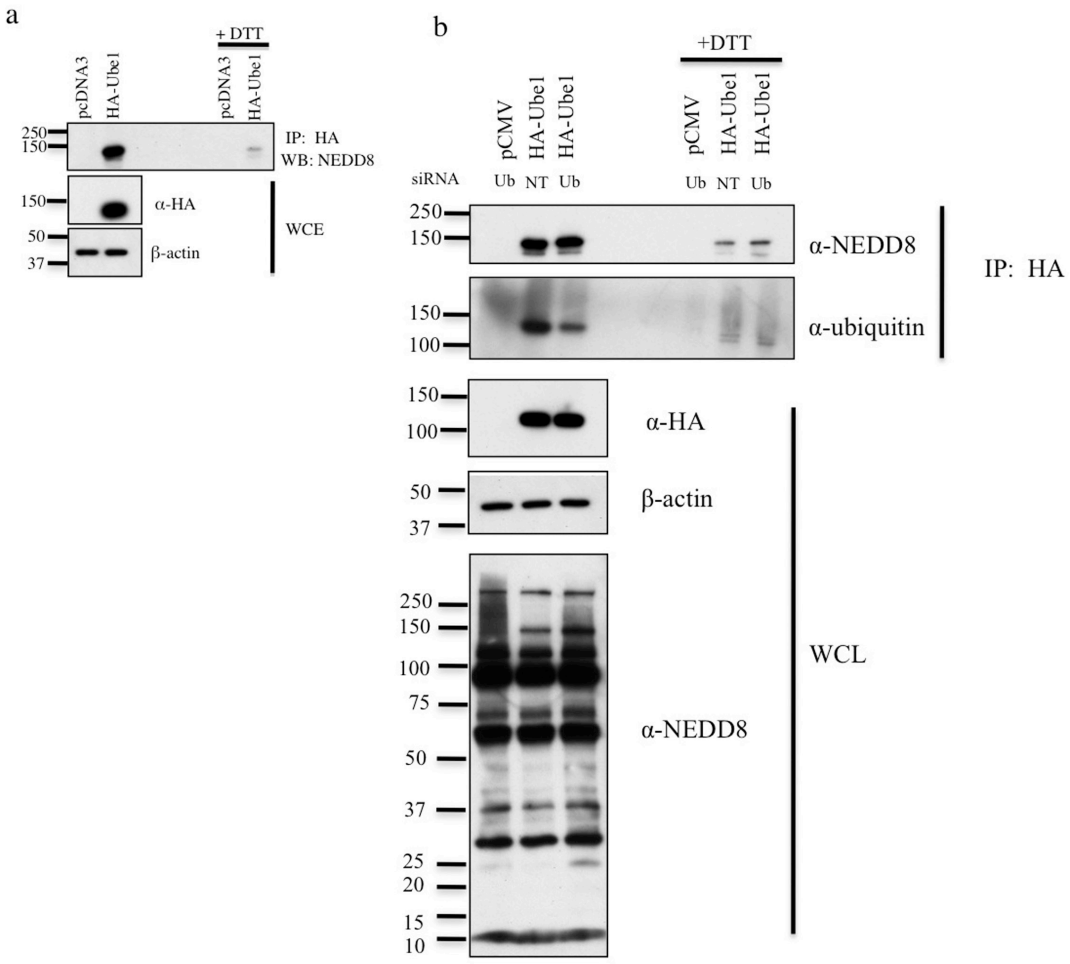


Figure 3.17: Ube1 forms a thiolester bond with NEDD8.

(a) U2OS cells were transfected with HA-Ube1 or pcDNA3 plasmid. 48 hrs later, cells were lysed as described in Materials and Methods. Anti-HA antibody coupled to sepharose beads was used for immunoprecipitation. After elution, samples were divided and in one set DTT (200 mM) was added before boiling. Proteins were analysed by western blotting. **(b)** Experiment was done as in Figure 3.17. **(a),** except HEK 293 cells were treated with non-targeting or ubiquitin siRNA for 15 hrs prior to immunoprecipitation.

3.2.9. p53 is NEDDylated by Ube1 under proteasome treatment

p53 has been shown to be modified by NEDD8, and this modification partially depends on the NAE under homeostatic conditions[102]. In this study, Xirodimas et al, 2004, the authors used a ts41 CHO cell line that has a temperature-sensitive NAE. At the restrictive temperature, p53 NEDDylation is largely decreased, but does not disappear completely (Figure 5. a in the paper). The authors' interpretation for the above result was that at the restrictive temperature some residual activity of the NAE allows partial p53 NEDDylation. In the context of our new finding that NAE is not the only E1 enzyme for NEDD8, we speculated that the Ube1 might be also responsible for p53 NEDDylation. We used a cell line stably expressing His_6-NEDD8 (6HisND8-MCF7) to assess the effect of Ube1 knockdown on p53 NEDDylation under MG132 treatment. Parental MCF7 cells or the stable cell line were treated with Ube1 siRNA or MLN4924, in combination with MG132. NEDD8 conjugates were isolated on Ni^{2+}-agarose beads, and analysed by western blotting (Figure 3.18).

NEDDylation of p53 was mainly dependent on Ube1 under proteasome inhibition. In contrast, the NEDD8 modification of cullin 3 was not affected by Ube1 knockdown, but was abolished when NAE was inhibited by MLN4924.

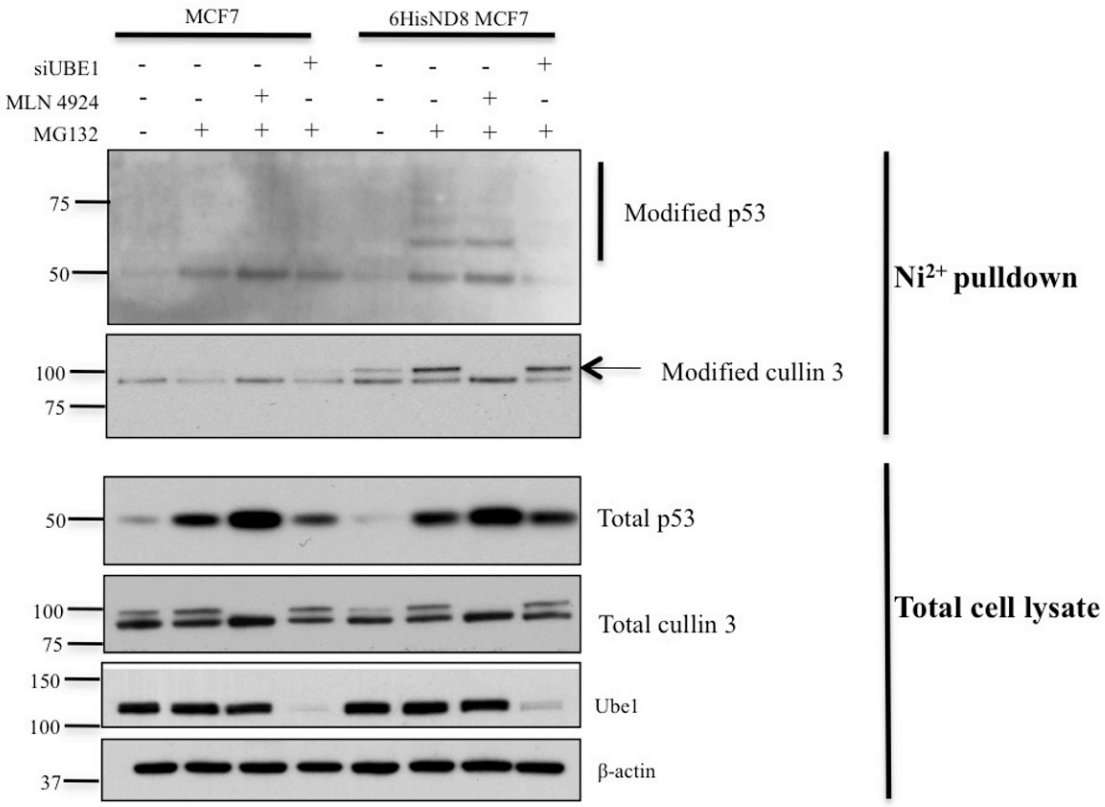


Figure 3.18: NEDDylation of p53 depends on Ube1 upon stress.

6HisND8 MCF7 cells were transfected with non-target (-) or with Ube1 siRNA for 48 hrs and treated with 1 μM MLN4924 for 16 hrs and 30 μM MG132 for 4 hrs as indicated. Cells were lysed under denaturing conditions and purification of His_6-NEDD8 conjugates was performed using Ni^{2+}-NTA agarose beads. Isolated proteins and total cell lysates were analysed with the indicated antibodies.

3.2.10. NEDD8 responds to heat shock in *C. elegans*, but not in *S. pombe*

We were interested to know whether NEDD8 responds to stress conditions only in human cell lines, or it is a conserved mechanism. Together with Aymeric Bailly we performed a heat shock experiment in *C. elegans*, where worms were shifted from 20^{o}C to 30^{o}C for 5 hrs and then total protein was extracted by bead beating and lysed as described in the Materials and Methods. Total worm extracts were analysed by western blotting with the human NEDD8 antibody. Since NEDD8 is highly conserved through species, we expected cross-reactivity with the worm NED-8. Indeed, we found that the antibody recognised NED-8, and the signal was increased in the heat-shocked samples, suggesting that the NEDD8 response to high temperature is a conserved mechanism (Figure 3.19 a).

We have also used *Schizosaccharomyces pombe* as a model organism to investigate the heat shock response. Contrary to *Saccharomyces cerevisiae,* ned8 deletion in fission yeast causes lethality. We used a wild-type strain (FY7093, genotype: h+, ade6-M210) for our investigation. Cultures were grown at 32°C (the optimal temperature for fission yeast growth), until they reached OD_{600} =0.3-0.6 (log phase, where the culture grows exponentially and is most susceptible to environmental changes). At that point, they were shifted to 37 or 39°C for the indicated time intervals. Total proteins were extracted as described in the Materials and Methods section, and analysed by western blotting with NEDD8 antibody from Epitomics. Again, we reasoned that due to the high conservation of NEDD8 through species, our antibody would recognize *S. pombe* ned8. It was indeed the case since a band corresponding to NEDDylated cullins was detected (Figure 3.19 b). However, we did not observed accumulation of higher molecular weight conjugates, as it was seen in human cell lines. Our western blot analysis with the

ubiquitin-specific antibody showed increase of ubiquitin conjugates at 1 hr after shifting to 37°C, but the same conjugates did not appear at later time points or higher temperatures. This preliminary data suggests that the NEDD8 heat shock response is not conserved between humans and *S. pombe*. However, we cannot exclude the possibility that we did not detect the response due to experimental issues. It would be interesting to investigate the same phenomenon in a strain that contains a tagged form of NEDD8, and use a tag-specific antibody. Alternatively, since NEDD8 has not been well-studied in *S. pombe* in response to stress conditions, further experimental conditions such as higher temperature (up to 42°C), or the use of the proteasome inhibitor (in a specific strain that can take up the drug) should be used to draw final conclusions.

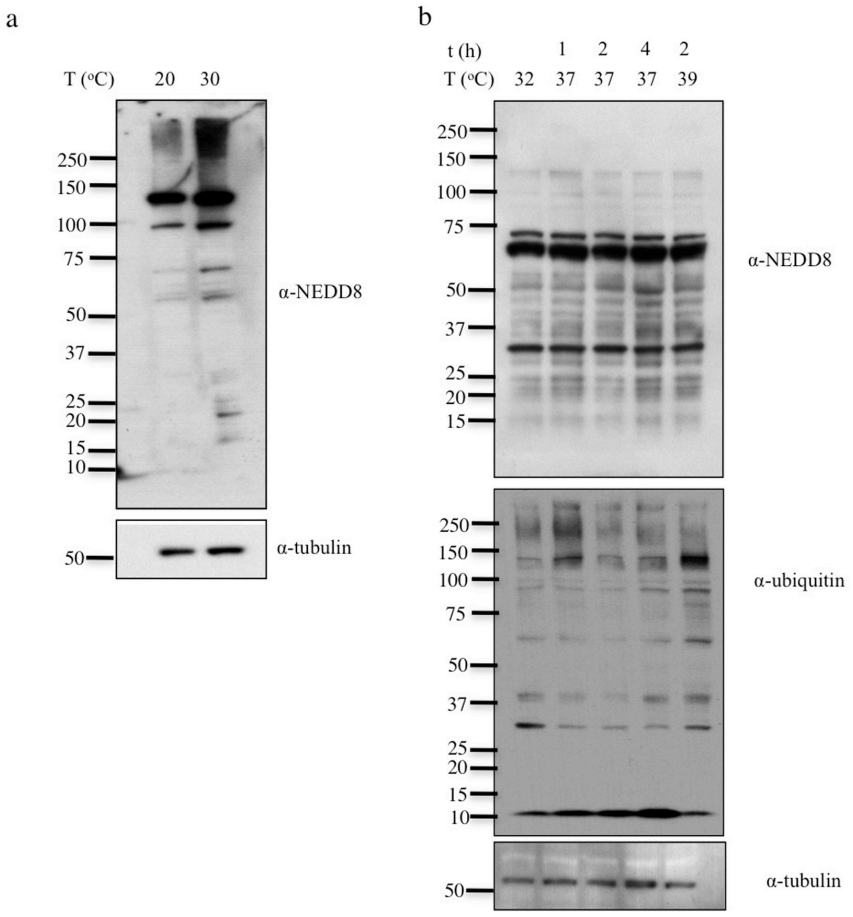


Figure 3.19: NEDD8 response to heat shock in *C.elegans* and *S. pombe*

(a) Synchronised worms were grown at 20°C and shifted to 30°C at L4 larvae stage. Total worm extracts were analysed by western blotting with NEDD8-specific antibody. **(b)** Wild-type *S. pombe* was cultured at 32°C or shifted to 37 or 39°C for the indicated time intervals. Total protein extracts were prepared as described in Materials and Methods, and analysed by antibodies specific to human NEDD8, ubiquitin and α-tubulin.

3.2.11. Characterization of NEDD8 surface residues required for Ube1-mediated activation by site-directed mutagenesis

We characterised the activation of NEDD8 by Ube1 in different cellular stress conditions. Next we aimed to address the biological outcome of this new type of NEDDylation. The NAE-dependent NEDDylation can be specifically inhibited by MLN4924, and the NEDD8 siRNA largely affects the conjugates that are dependent on Ube1. The remaining NEDD8 appears to be efficient to modify cullins, and allows almost intact functioning of the CRL E3 ligases, as determined by the detection of two substrates, cyclinE and p21 (Figure 3.20). SCF ligases normally degrade these two proteins; however, inhibition of cullin NEDDylation by MLN4924 results in an increase of cyclinE and p21. On the other hand, partial NEDD8 knockdown does not have a major affect on cullin NEDDylation, and importantly, does affect SCF activity, as SCF substrates do not stabilize. However, complete inhibition of the Cullin Ring Ligases by MLN4924 perturbs the ubiquitin pathway, which is also engaged in NEDD8 conjugation under certain conditions, therefore this drug is not an appropriate tool to investigate the roles of the two types of NEDDylation.

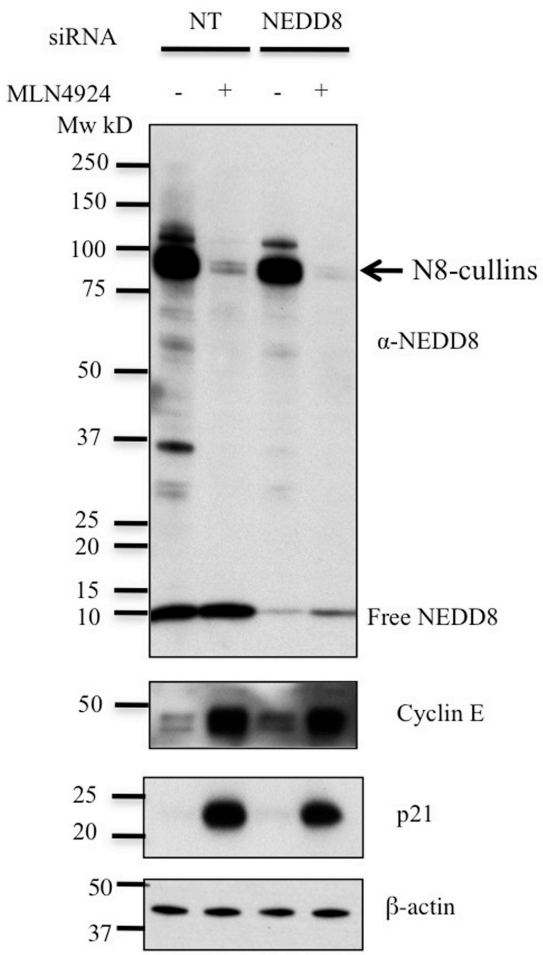


Figure 3.20: NEDD8 knockdown does not affect cullin NEDDylation and function of the SCF-ligase complex.

The upper panel is a part of Figure 3.4. The same extracts were blotted against substrates of the SCF-ligase complex. While MLN4924 treatment inhibits cullin NEDDylation and allows accumulation of cyclin E and p21, NEDD8 knockdown does not affect the function of the SCF-ligase complex.

To find a tool that will distinguish between the two pathways, the NAE- and the Ube1-dependent NEDDylation, we decided to search for a NEDD8 mutant that is activated by NAE but not by the Ube1. Such mutant could be used to replace wild type NEDD8 in human cell lines or in a model organism and allow testing the role of atypical NEDDylation on cell survival or response to stress.

We were aiming to identify the residue(s) on NEDD8 that is required for the activation by Ube1. For this, we set up the following criteria:

1. The amino acid has to be a surface residue

2. Should not be required for NAE-mediated activation [97]

Amino acids that have been published in Walden et al., as shown in Figure 3.21, to be in contact with NAE during activation were not included in our screen.

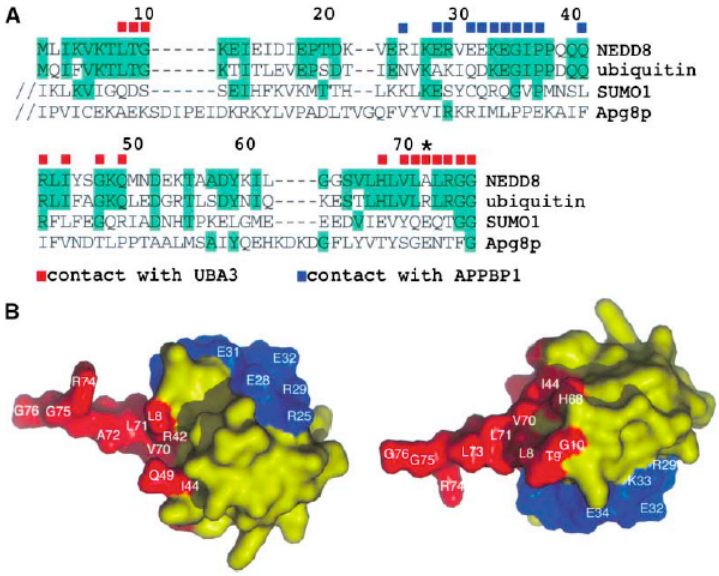


Figure 3.21: Residues on NEDD8 required for the interaction with NAE

(a) Sequence alignment of NEDD8, ubiquitin, SUMO1 and *S. cerevisiae* Apg8p with the identical residues to NEDD8 are shaded and the structure of NEDD8 **(b)** are adapted from[97]. The residues highlighted in red are in contact with UBA3. The residues in blue are in contact with APPBP1.

3. Should be conserved between humans and *S. cerevisiae*

Since it has been published that Ube1 activates overexpressed NEDD8 (Rub1) in *S. cerevisiae*[260], we reasoned that the essential amino acid is most likely conserved between the two organisms (Figure 3.22). To make our mutational analysis easier, we eliminated the non-conserved amino acids from our screen.

```
                *::**********.:::: :* * :*** :********.*****:.***::*: *.:* ::: *  *****:****.
Rub1p           MIVKVKTLTGKEISVELKESDLVYHIKELLEEKEGIPPSQQRLIFQGKQIDDKLTVTDAHLVEGMQLHLVLTLRGGN
NEDD8           MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMNDEKTAADYKILGGSVLHLVLALRGGG
                1.......10........20........30........40........50........60........70.......
```

Figure 3.22: ClustalX sequence alignment between Rub1p (accession number: NP_010423.2) and NEDD8 (accession number: NP_006147)

An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties. A . (period) indicates conservation between groups of weakly similar properties. The residues are coloured according to their physicochemical properties (red: small + hydrophobic; blue: acidic; magenta: basic; green: hydroxyl + sulfhydryl + amine + G; grey: others).

Figure 3.23. shows the structure of NEDD8 (PDB ID number 1NDD from PDBTM) that was modified in MacPyMOL software to highlight the residues selected for site-directed mutagenesis. S46 and V66 were later added to the list, based on previous observations that these mutants showed unexpected NEDDylation patterns.

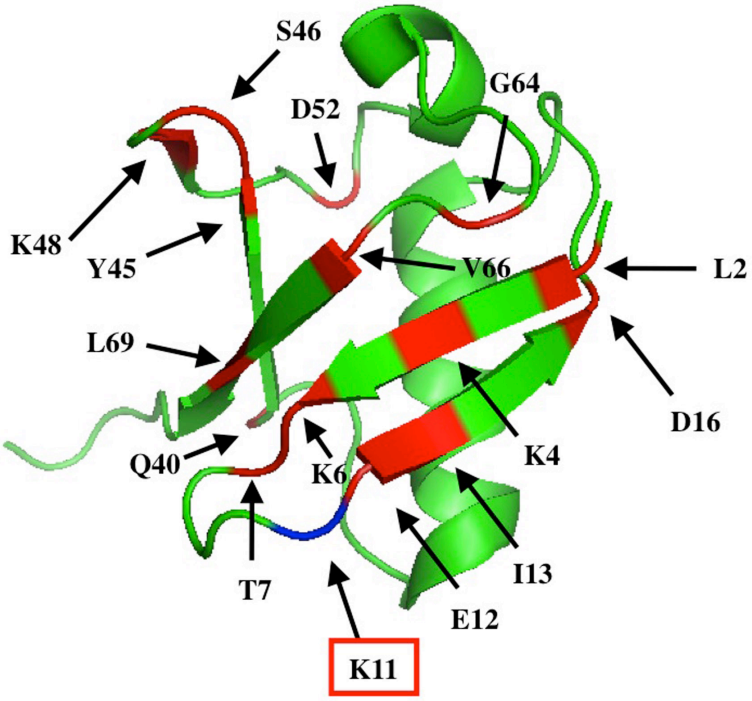


Figure 3.23: The residues on NEDD8 selected for mutagenesis.

Ribbon view of the three-dimensional structure of NEDD8 was generated by PyMOL v0.99. The selected amino acids are highlighted in, and the K11 residue in blue.

The mutageneses to alanine (or arginine in the case of the lysine residues) were performed on pcDNA3 HA-NEDD8 vector, followed by verification by sequencing. The plasmids were overexpressed in H1299 cells, treated or untreated with MG132, and lysed directly with 2X SDS. The NEDDylation pattern was visualised by Western blotting, with HA-antibody. The conjugation profile was compared to that of the wild-type HA-NEDD8. Figure 3.24-25. show a few representative examples of the tested mutants.

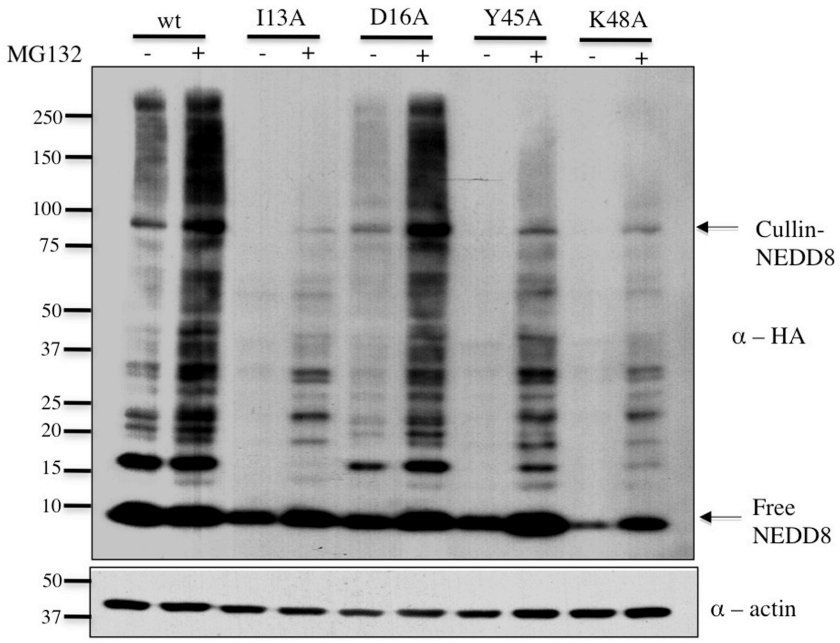


Figure 3.24: Examples of the behaviours of selected NEDD8 mutants.

H1299 cells were transfected with wild type or mutant NEDD8 constructs. 48 hrs after transfection, cells were treated or not with 30 μM MG132 for 4 hrs, directly lysed in 2X SDS, and analysed by western blotting with the antibody specific for the HA-tag.

We were looking for a mutant that fulfills the following criteria:

- expressed similarly to wild type NEDD8;
- can be conjugated to cullins, the well-known NAE-dependent substrates;
- the increase of the NEDD8 conjugates after MG132 treatment is impaired.

The K11R mutant appeared to fulfill these criteria: it was expressed, was conjugated to cullins, but the MG132 response was impaired. We have seen the same effect when we mutated Lys11 to alanine. No other mutant showed the same phenotype.

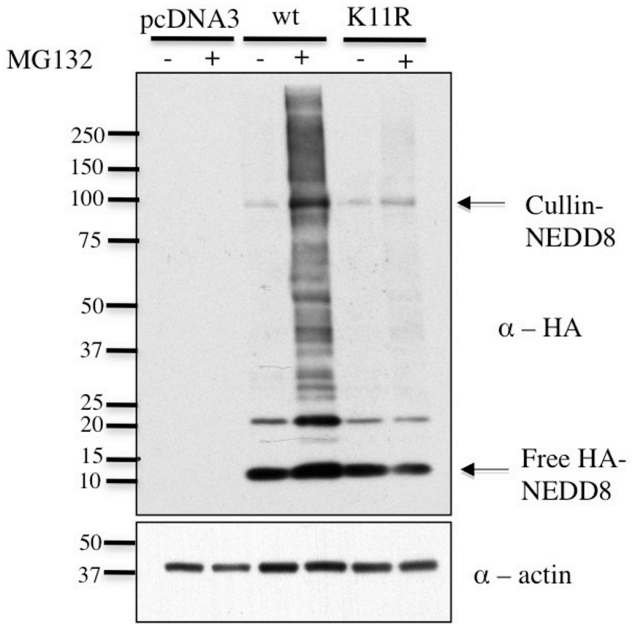


Figure 3.25: NEDD8 K11R mutant does not respond to MG132 treatment.

H1299 cells were transfected with wild type or K11R mutant NEDD8 constructs. 48 hrs after transfection, cells were treated or not with 30 μM MG132 for 4 hrs, directly lysed in 2X SDS, and analysed by western blotting with HA-specific antibody.

Table 3.1 contains the summary of the mutations and their behavior in response to MG132 treatment.

Mutation	Expression	Cullin NEDDylation	MG132 response
L2A	✓	✓	✓
K4A	✓	✓	✓
K6A	✗	✗	✗
T7A	low	weak	✓
K11A	low	weak	✗
E12A	✓	✓	✓
I13A	low	weak	✓
D16A	✓	✓	✓
Q40A	✓	✓	✓
Y45A	✓	✓	✓
K48A	✓	✓	✓
D52A	✓	weak	weak
G64A	✓	weak	weak
L69A	low	✓	✓
S46A	✓	✓	✓
V66A	✓	✓	✓
K6R	low	✓	✓
K11R	✓	✓	✗

Table 3.1: Summary of the NEDD8 mutants used in the screen

These preliminary results show that K11 on NEDD8 could be potentially important either for the activation by Ube1, or for the chain formation.

3.3. Discussion

Interplay between posttranslational modifications provides complex and diverse ways of regulating the proteome. It has been well documented that one ubl can be responsible for the regulation of other ubl conjugation machinery: Cullin RING Ligases are activated through NEDDylation, and SUMO chains signal for ubiquitination and proteasomal degradation of certain proteins.

In this project we have discovered an unprecedented cross-talk between NEDD8 and ubiquitin conjugation pathways. We found that upon diverse stress conditions including proteasome inhibition, heat shock and oxidative stress NEDD8 conjugation dramatically increases in cells. Surprisingly however, NEDD8 conjugation does not depend on the NEDD8 activating E1 enzyme NAE but rather on the ubiquitin E1 activating enzyme Ube1. The observation that Ube1 can activate NEDD8 and transfer it to ubiquitin E2s[260], together with the current knowledge on shared E3 ligases between NEDD8 and ubiquitin pathways[102, 103] fundamentally changes our understanding about Ubl conjugation pathways. Redundancy between the cascades exists in a way that NEDD8 can be used by two distinct pathways: through NAE that mainly controls cullin NEDDylation and through the Ube1 pathway that expands conjugation of NEDD8 to substrates that are also ubiquitinated. While our data show that the later mechanism is dramatically induced under diverse cellular stress conditions, we cannot exclude the possibility that conjugation of NEDD8 through Ube1 occurs under unstressed conditions.

It is tempting to draw a paralell between SUMOylation and NEDDylation. The SUMO family consists of three members: SUMO-1 and SUMO-2,3. SUMO-1 shares only about 50 % sequence identity with SUMO-2/3, which means it is less identical to the other SUMO members than NEDD8 is identical to ubiquitin. The three SUMO

members share the same E1 activating enzyme. SUMO-1 modification appears to play role in protein regulation under homeostatic conditions. Its main role is the regulation of RanGAP1 through covalent modification that promotes RanGAP1 association with the nuclear envelope, to fulfill its function in nuclear protein import[159]. Recently SUMO-1 modification on PTEN was found to be important to increase PTEN binding to the plasma membrane, which is required for its tumor suppressor activity[264]. To our knowledge, SUMO-1 fulfills its regulatory roles by modifying its targets in a monomeric form. On the other hand, SUMO-2 and-3 appear to be stress-responsive regulators of the proteome, as it was demonstrated upon heat shock and proteasome stress; and they mainly represent a signal in a form of polySUMO chains. To this analogy, NAE-mediated NEDDylation that mainly regulates Cullin RING Ligases resembles homeostatic modification by SUMO-1. On the other hand, the Ube1-mediated NEDDylation, similarly to SUMO-2/3 modifications, is engaged in protein modification under stress conditions. The utilization of one posttranslational modifier, NEDD8 through two distinct pathways in order to suit diverse cellular functions makes it a very versatile regulator of protein function. This is a good example of how the complexity of the proteome can be increased by modification through different ubiquitin-like conjugation pathways to compensate for the low number of genes in vertebrate genomes.

We showed the first *in vivo* evidence that NEDD8 does not only respond to pharmacological inhibition of the proteasome, but also to environmental stress conditions such as heat shock or oxidative stress. In all tested conditions, Ube1 mediated the NEDD8 activation. We also gained evidence on the NEDD8 response to heat shock in *C. elegans*. Very recently, Lee et al. has demonstrated a global NEDD8 accumulation during torpor in the brains of hibernating ground squirrels by western blot analysis[265]. This study suggests a role for NEDD8 in ischemic stress. Even though

the nature of this increased NEDD8 conjugation was not addressed in the paper, their western blot analysis clearly resembles the pattern of the Ube1-mediated NEDD8 response to heat shock or oxidative stress. Taken together, NEDD8 seems to act as a general stress response molecule to diverse environmental challenges in different organisms.

In this project we also gained a mechanistic insight of Ube1-mediated NEDDylation. Heat shock results in exhaustion of the unconjugated ubiquitin pools by generating misfolded proteins, that are excellent substrates for the UPS (ubiquitin-proteasome system)[11, 266]. Although it is still a matter of debate to what extent the ubiquitin-proteasome system is involved in eliminating oxidized proteins, there is increasing amount of studies showing the response of the ubiquitin pathway to oxidative stress [267]. What is also evident is that under these stress conditions the proteasome activity may also be compromised through depletion of ATP (heat shock) or due to dissociation of the 20S core particle from the 19S regulatory particle (oxidative stress) [268, 269]. A common phenomenon under conditions of proteasomal activity perturbations is the depletion of free ubiquitin. Under oxidative stress conditions our data show that while ubiquitin conjugates are not dramatically affected, the level of free ubiquitin is decreased at 1 mM H_2O_2 treatment. This is the exact concentration where NEDDylated proteins accumulate in a Ube1-dependent fashion. These observations led us to hyphothesise that the changes in the relative levels between NEDD8 and ubiquitin will provide a trigger for Ube1 to activate NEDD8. We show that a decrease in the ubiquitin levels (by siRNA treatment) in the absence of any additional stress, was sufficient to stimulate NEDD8 conjugation through the ubiquitin E1 activating enzyme. We also tested whether ubiquitin knockdown affects general SUMOylation, a posttranslational modification that responds to the same stress conditions. SUMO did not accumulate upon ubiquitin knockdown, suggesting the existence of differential mechanisms for

NEDD8 and SUMO to respond to cellular stress. In support of the model that the relative levels of NEDD8 to ubiquitin control the activation of NEDD8 by Ube1, Hjerpe et al. show that overexpression of NEDD8 results in activation and conjugation through the ubiquitin pathway[260]. Additionally, we speculate that an active switch may exist to allow activation of NEDD8 by Ube1 *in vivo* under stress conditions. This is supported from the observation that ubiquitin overexpression cannot rescue the NEDD8 response (Figure 3.16). There are other experimental indications that the control of Ube1-mediated NEDD8 activation is more complex than the ratio between free ubiquitin/NEDD8 and Ube1. As discussed in Chapter 3.2.8 , Figure 3.17b shows that Ube1 overexpression alone is sufficient to allow thiolester formation between the enzyme and NEDD8, however, it does not result in NEDD8 conjugation in the absence of stress. Ube1 and NEDD8 are posttranslationally modified, which may increase the affinity of Ube1 for NEDD8, allowing its activation and conjugation by the ubiquitin pathway[270-272]. This would explain the poor activation of NEDD8 by Ube1 *in vitro*, where recombinant proteins are used[261].

One possible interpretation for the activation of NEDD8 by Ube1 could be that NEDD8 and ubiquitin are used in a redundant way: when cells run low on ubiquitin, it substitutes with a similar modifier. Against this argument is the observation that the yeast polyubiquitin gene is induced upon heat shock and starvation to increase cell resistance to stress [11]; indicating that organisms do produce more ubiquitin, if they need so. However, such induction in human cells does not happen, instead, -based on our observations- the system prefers to involve a different modifier in the response. NEDD8, despite its close homology with ubiquitin has distinct biophysical and thermodynamic properties from ubiquitin. NEDD8 appears to be more thermolabile and more prone to unfolding than ubiquitin[273]. These characteristics might be biologically significant if NEDD8 modification on proteins is recognized and handled differently to

ubiquitin signals. For instance, changes in rates of degradation could give more opportunities for refolding of misfolded proteins, which is an energetically favourable mechanism in higher eukaryotes. Alternatively, the increase in NEDD8 conjugation observed under stress conditions could be part of a mechanism to dampen the depletion of ubiquitin and part of a response to restore the initial ubiquitin levels.

Another interesting observation is that the free NEDD8 is not depleted, while the levels of NEDD8 conjugates increase. It would be important to test whether the reamining unconjugable NEDD8 is processed, or this pool consists of freshly translated, unprocessed form of the ubl. Is the NEDD8 processing somehow inhibited under stress conditions? It would be also interesting to test the subcellular localization of these NEDD8 species. It has been noted that ubiquitin mainly exists in the cells already charged on the E1 enzymes (Millenuim Pharmacheuticals, personal communication). It is possible that the free NEDD8 we observe on the western blot after cell lysis under reducing conditions is actually thiolester-bound NEDD8 in the cells, waiting for a trigger to be quickly transferred to E2s and substrates when a prompt response is required.

To draw a conclusion on the biological significance of the Ube1-mediated NEDDylation, we need to distinguish between the two pathways in an unambigous manner. The last part of the project, which is in progress, is aiming to develop a system that makes this differentiation possible. By site-directed mutagenesis, we identified a residue on NEDD8, Lys11, that is likely to be required either for the NEDD8 activation by Ube1, or for chain formation. Diglycine modification on NEDD8 K11 residue has been identified by MS in previous studies[44, 107]. However, this is not the only linkage through which NEDD8 can form chains. The K11 residue can be important for the activation by Ube1, therefore in the future we aim to test whether the K11R mutant can form a thiolester bond with Ube1. Alternatively, it is possible that the activation by

the ubiquitin cascade is initiated by a NEDD8 dimer (or other intermediate) formation through K11 linkage. Once this intermediate is formed, NEDD8-ubiquitin chain can be built up rapidly through different linkages. This hypothesis is to be tested, but there is evidence suggesting that Ube1 forms thiolester mainly with NEDD8 in a dimeric form ([260] and our observations). It would be interesting to immunoprecipitate NEDD8 with Ube1, break up the thiolester linkage, and identify the nature of the dimer by mass spectrometry.

Utilization of a NEDD8 mutant that can only be activated by NAE will allow us to gain evidence on the biological significance of the Ube1-mediated NEDDylation and the exact cellular processes it is involved in.

Chapter 4: Characterization of NEDD8-ubiquitin chains *in vivo*

4.1. Introduction

Mass spectrometry (MS) is an ideal technique for examining chemical modifications, such as posttranslational modifications of proteins. By measuring the mass-to-charge ratios of a modified peptide and its fragment ions, MS is unique in presenting direct evidence of modification sites[274]. Thus, it provides the crucial starting point for determining the biological significance for the post-translational modification in question. This includes the creation of site-specific mutations on the substrate of interest. Phosphoproteomics has been particularly successful, and more recently, similar approaches have been developed to detect proteins/peptides modified by ubiquitin/ubls.

For large-scale identification of ubiquitinated proteins, due to their low abundance, it is necessary to purify them for enrichment. This can be done on the protein or peptide levels. Purification of proteins relies on the usage of cell lines that express non-endogenous tagged ubiquitin/ubl. The most widely used epitope tags include the short histidine stretches (eg.6His-Ub/NEDD8), where purification is done under denaturing conditions with metal ions, such as Ni^{2+} or Co^{2+}. Immunoprecipitations through Myc-tag, FLAG-tag or HA-tag are also popular. However, the native conditions used for the purification preserve non-covalent interactions, therefore covalent modifications have to be confirmed by the detection of the site of modification[275]. Tandem-affinity purification strategies (with TAP-tagged proteins) have been originally developed to study non-covalent protein-protein interactions. By introducing a lysis step under denaturing conditions prior to purification, it can be also applied to study proteins modified by ubls. This approach reduces contaminations resulting from non-covalent interactions, and at the same time preserves ubl-modifications by inhibiting proteases[276].

Ubiquitin-binding domains can be also used to enrich substochiometrically modified proteins under endogenous conditions. Reagents consisting of tandem ubiquitin binding domains have been developed for more efficient pulldowns[277, 278]. SUMO conjugates have been also purified with tandem SIM (SUMO-interacting motif) domains[279].

Trypsin, the enzyme widely used in proteomics studies to digest proteins, cleaves at the C-terminus of arginine and lysine (unless the lysine is modified or followed by proline residues). In the case of ubiquitinated proteins, there is no cleavage at the modified lysine, and the mass of the peptide is increased by the two glycines that remain from the digested ubiquitin. This provides a predictable mass shift of the precursor ion (+114.0429), and allows detection of peptides modified by ubiquitin[280].

A recently published monoclonal antibody that recognizes the isopeptides that contain the diglycine motif provides a powerful tool to study ubiquitination on the peptide level, making it possible to identify the site of modification[281]. This is critical when a protein is modified on multiple sites. In the recent years several different laboratories utilized the GlyGly antibody for proteomics studies[282-284]. In the largest study, around 19,000 diglycine-modified peptides within 5000 proteins have been identified[44]. The proteomics data have been deposited in a publicly available database, and can be a basis of further biological studies.

However, ubiquitin is not the only modification that leaves the diglycine signature on lysine residues: tryptic digestion of NEDD8 and ISG15 provide the same mass shift. Due to the increasingly important regulatory roles of ubls and the several examples of crosstalks between the modifiers, methodological advances that distinguish unambiguously between ubiquitin and NEDD8 sites have to be developed in the near future[280].

Apart from substrate identification, monitoring of the ubiquitin/ubl modified proteome upon cellular stimuli or in response to pharmaceutical agents is crucial to understand protein regulation by these pathways. However, mass spectrometry is not inherently quantitative. In order to monitor changes in the proteome upon stimuli, absolute or relative quantitation approaches can be used. The AQUA approach uses stable isotope labeled peptides as internal standard to study complex biological samples quantitatively[285]. On the other hand, the popular SILAC method (Stable isotope labeling by amino acids in cell culture) provides relative quantification between two cell populations, at the protein levels[286]. It uses the metabolic labeling of proteins with normal or heavy amino acid isotopes. Typically, two cell populations are grown in culture media that only differ from the form of a particular amino acid (in the heavy medium, arginine and lysine are replaced by their ^{13}C and/or ^{15}N carbon labeled forms, leading to C-terminal labeling of tryptic peptides). The incorporation of these amino acids into peptides lead to a known mass shift that is detectable by MS[287].

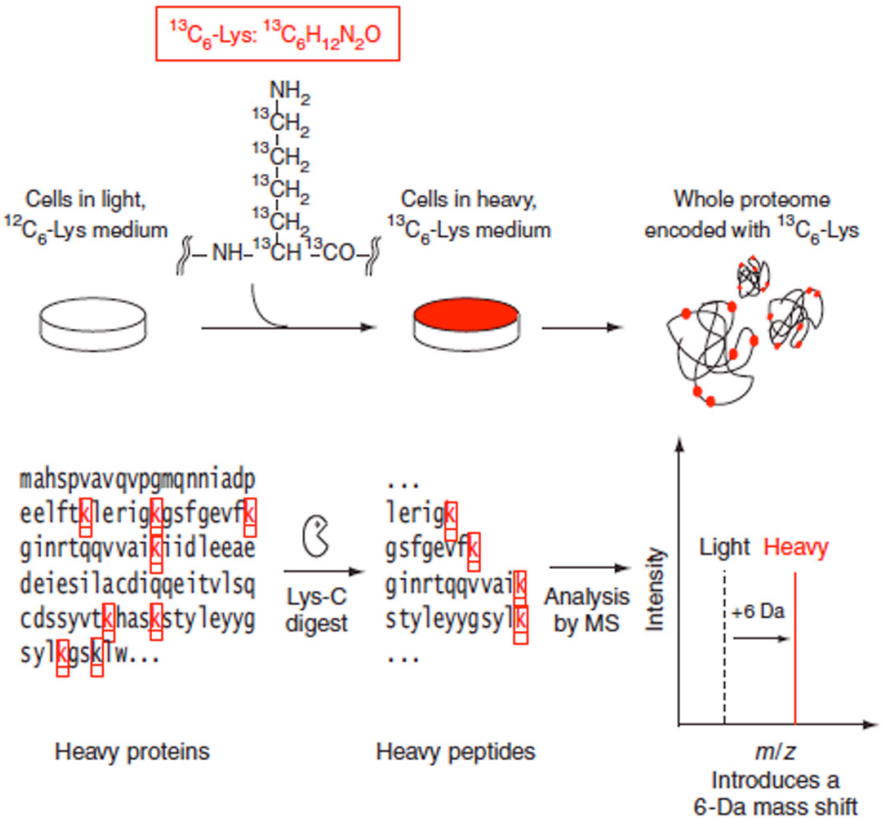


Figure 4.1: An example of a SILAC workflow.

^{13}C labeled lysine incorporation throughout the proteome followed by Lys-C digestion results in a predictable mass shift of 6 Da from the light peptide. Adapted from:[288]

We have shown that the ubiquitin E1 enzyme activates NEDD8 under certain conditions. An important question is the nature of the chains formed on substrates when the ubiquitin pathway uses two different modifiers at the same time. Based on *in vitro* [102, 261] and *in vivo* data[102, 289], substrates can be simultaneously modified with NEDD8 and ubiquitin. What is also evident is that within this mixture both NEDD8 and ubiquitin are modified, however the nature of formed chains has been unclear.

In this project we investigated the nature of the chain formation under proteasome inhibition, and the recognition of the signal by ubiquitin-binding domains.

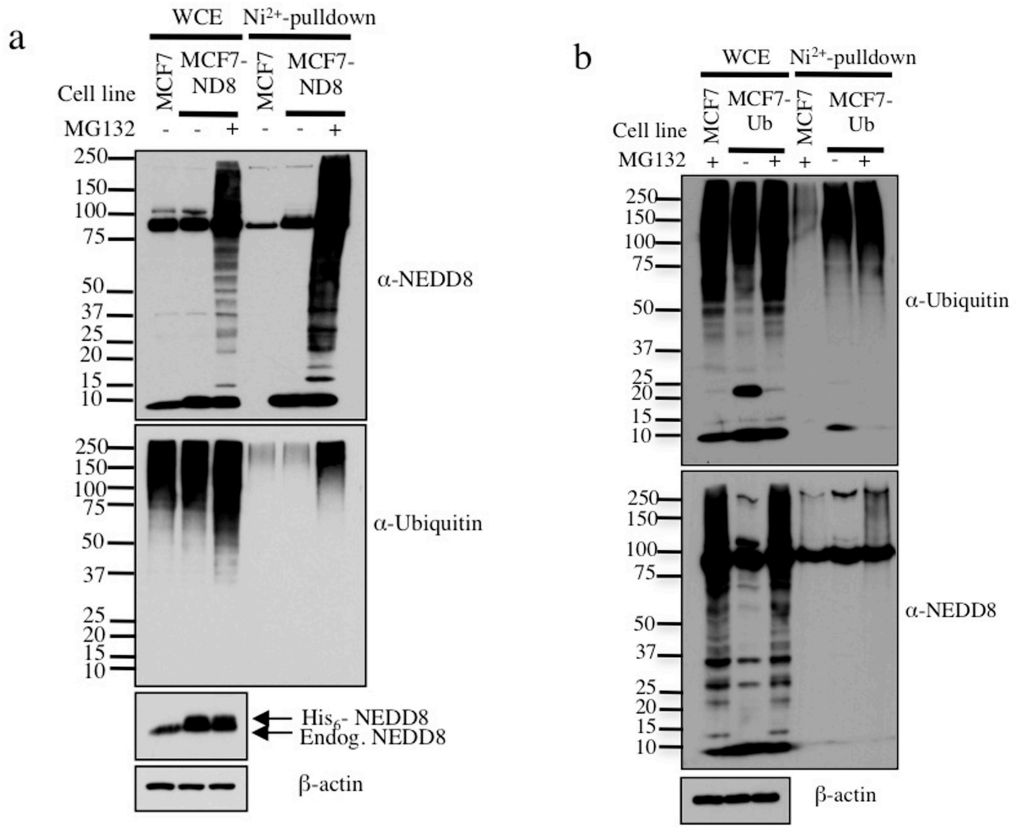
4.2. Results

4.2.1. NEDD8 and ubiquitin coexist on substrates

Our data demonstrated that pharmacologic inhibition of the proteasome causes a dramatic increase in NEDD8 conjugation which involves the ubiquitin conjugation pathway. We addressed the nature of these conjugates and more specifically the presence of ubiquitin. For this we used MCF7 cells stably expressing His_6-NEDD8 (MCF7-ND8) at levels similar to endogenous NEDD8 [142, 290]. Cells were either untreated or treated with MG132 and NEDDylated proteins were isolated under denaturing conditions using Ni^{2+} agarose beads to prevent any noncovalent interactions. Similarly to what is observed in parental MCF7 cells, MG132 treatment causes a dramatic increase of NEDD8 conjugates in the MCF7-ND8 cells (Figure 4.2.a). We found that upon MG132 treatment the accumulated NEDD8 conjugates are also enriched with ubiquitin suggesting that upon stress conditions substrate proteins can be simultaneously modified with NEDD8 and ubiquitin. Next we performed the reverse experiment: we isolated His_6-ubiquitin conjugates on Ni^{2+}-beads, and detected the conjugates with ubiquitin and NEDD8 antibodies. Indeed, NEDD8 was present among the ubiquitinated substrates under MG132 treatment, confirming that the two posttranslational modifiers can simultaneously modify proteins (Figure 4.2.b).

We also looked at the endogenous NEDD8 conjugates. In proteomic approaches, where the aim is the identification of ubiquitin conjugates, pulldowns with proteins containing ubiquitin-binding domains (UBDs) or with the domain itself are frequently used[275]. We have expressed the recombinant UBA-domain of Dsk2 (the *S.cerevisiae* homologue of HsPlic2), and coupled to sepharose beads to isolate endogenous ubiquitin conjugates. MCF7 cells were treated or not with MG132 and the lysate was incubated with

recombinant UBA. The domain efficiently isolated ubiquitin conjugates, among which we detected NEDD8 signal as well from the MG132 treated samples (Figure 4.2.c). This result indicates that endogenous NEDD8 is present on ubiquitinated substrates.



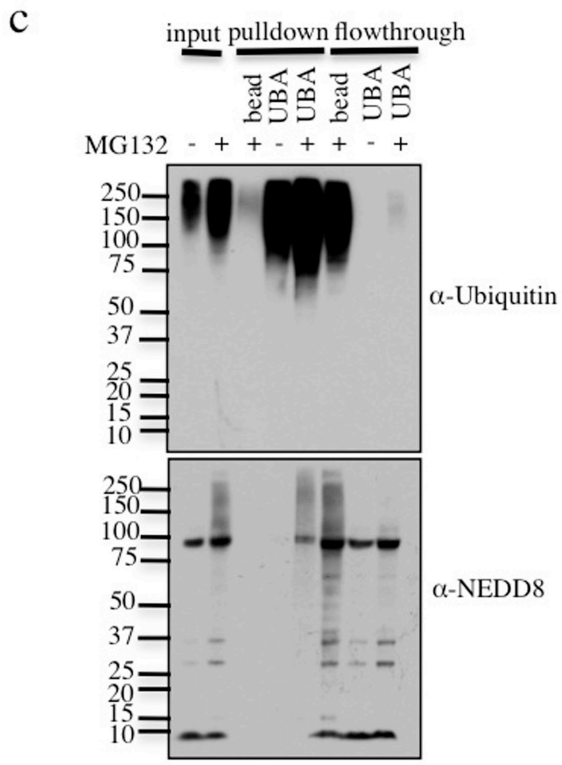


Figure 4.2: Ubiquitin and NEDD8 co-precipitate in different pulldown strategies.

(a) Parental MCF7 or MCF7 cells stably expressing His_6-tagged NEDD8 (MCF7-ND8) were left untreated or treated with 30 μM MG132 for 4 hrs. NEDDylated proteins were isolated as described in the Materials and Methods, using Ni^{2+}-NTA agarose beads. Whole cell lysates or purified proteins were analysed by western-blotting, using NEDD8 or ubiquitin antibodies. **(b)** Experiment was done as described at Figure 4.2.a, except using MCF7 cells stably expressing His_6-tagged ubiquitin. **(c)** Pulldown of ubiquitinated proteins was performed by using recombinant UBA domain derived from Dsk2 protein. The eluates and flowthrough were analysed by western blotting.

4.2.2. Ubiquitin and NEDD8 chains are enriched in the NEDDylated fraction

Xirodimas et al., 2008 employed a SILAC method to label HeLa cells stably expressing TAP-NEDD8 with medium containing either ^{12}C-Arginine or ^{13}C-Arginine and cells were either untreated (^{12}C) or treated with MG132 (^{13}C). As Figure 4.3.a shows, cell extracts were mixed in 1:1 ratio, followed by a tandem affinity purification, in gel digestion with trypsin and mass spectrometric analysis[106]. We re-analysed these data using MaxQuant software[249]. We performed a quantitative analysis on the NEDD8 modified peptides upon MG132 treatment, by looking for NEDD8 peptides that contain diglycine modification on lysines. The employed labeling method allowed quantification of the abundance of the NEDD8 linkage at K22 and a previously *in vivo* unreported linkage at K33. The analysis showed that conjugation at both sites increases upon MG132 treatment (Figure 4.3.b,c and Figure 4.4. a-c) [106, 107]. We also identified and quantified ubiquitin peptides within the NEDD8 conjugates with diglycine modification on K48 and K63. The analysis showed that ubiquitin chains are also present in the NEDD8 pulldown and increase upon MG132 treatment (Figure 4.3.d,e and Figure 4.4. d, e). These results indicate that treatment with MG132 causes an increase in NEDD8 and ubiquitin chain formation.

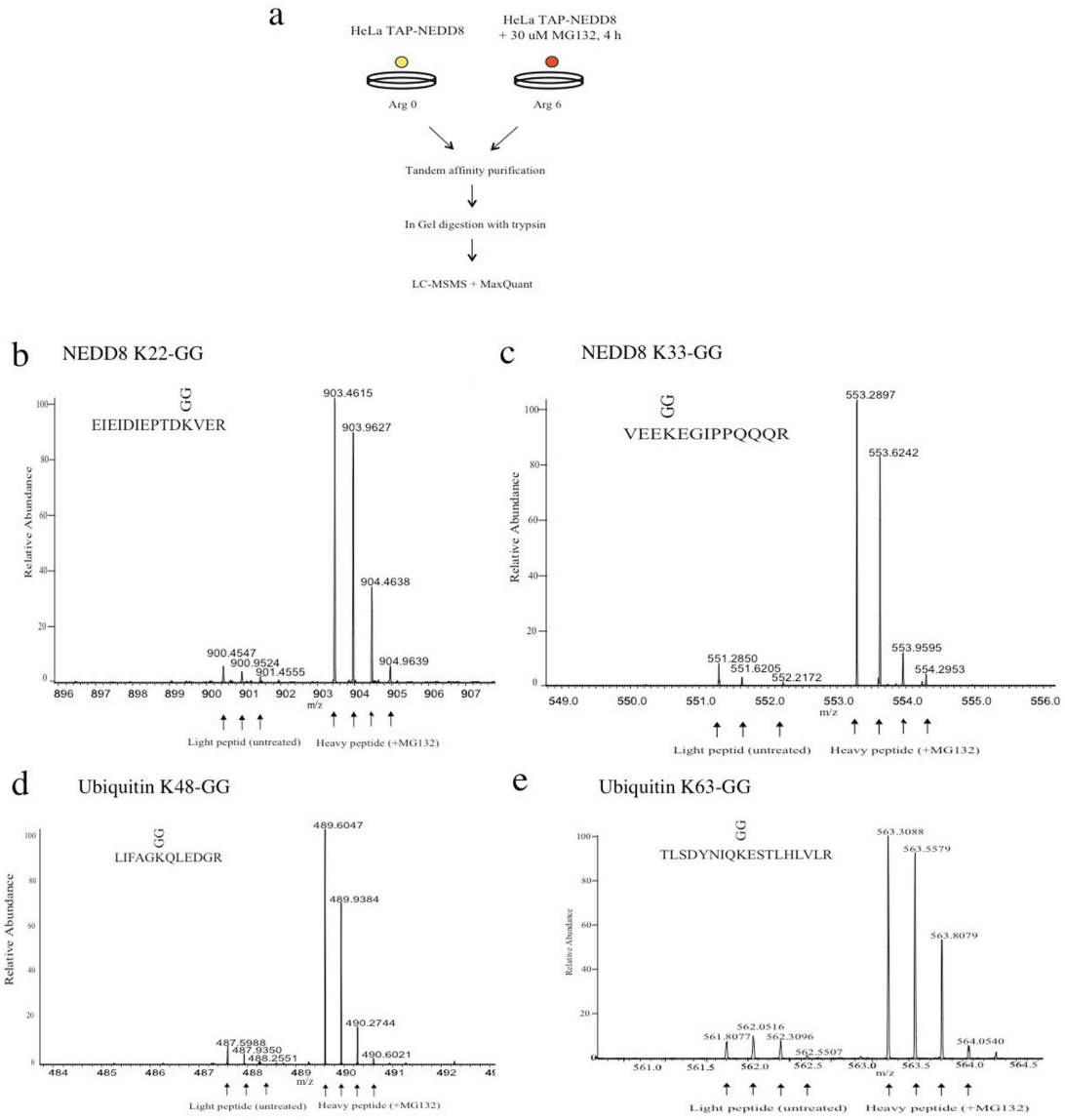
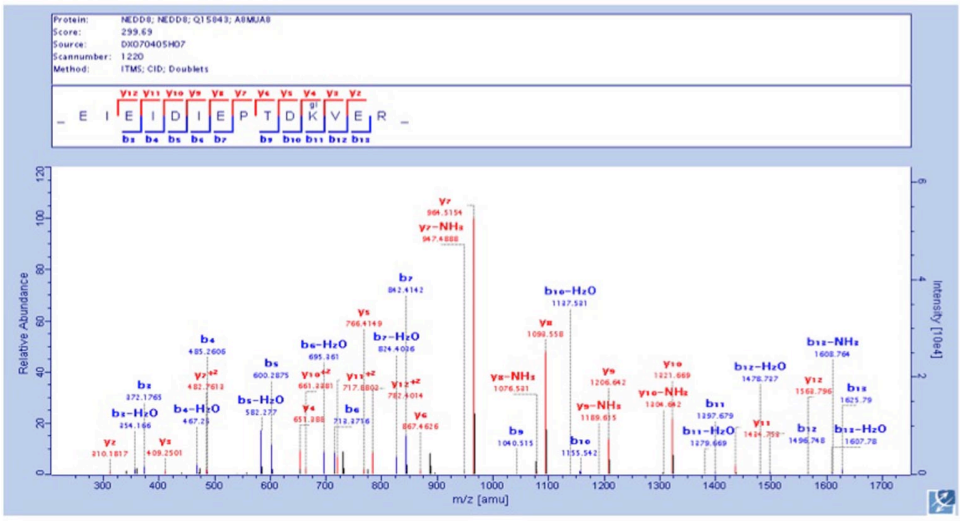


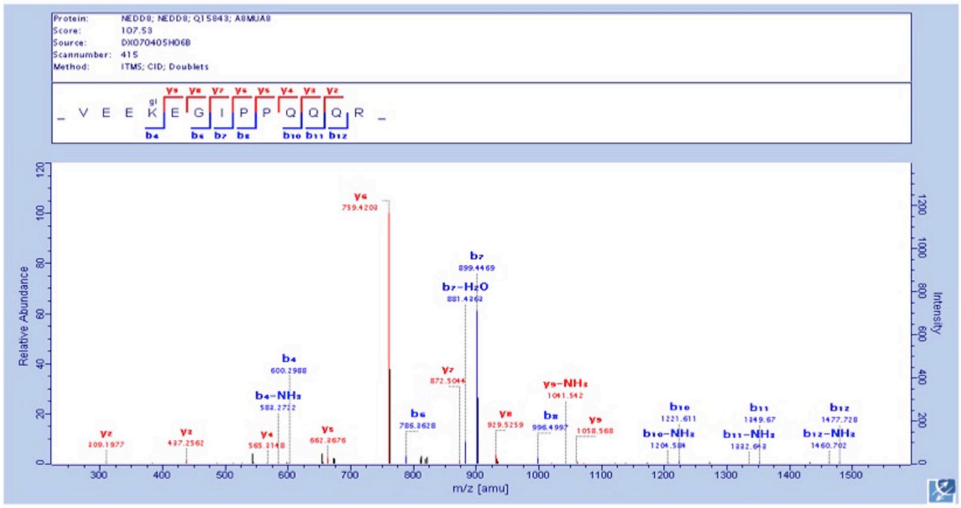
Figure 4.3: Diglycine modified ubiquitin and NEDD8 peptides are enriched upon MG132 treatment within the NEDD8 proteome.

(a) Schematic representation of the SILAC workflow. The data from a SILAC experiment performed in Xirodimas et al., 2008 were re-analysed using MaxQuant (see main text for details). MS spectra of NEDD8 peptides modified by Gly-Gly on K22 **(b)** or on K33 **(c)** and ubiquitin peptides modified on K48 **(d)** and K63 **(e)**. The SILAC ratio indicates an increase of the modification for all peptides with MG132 treatment.

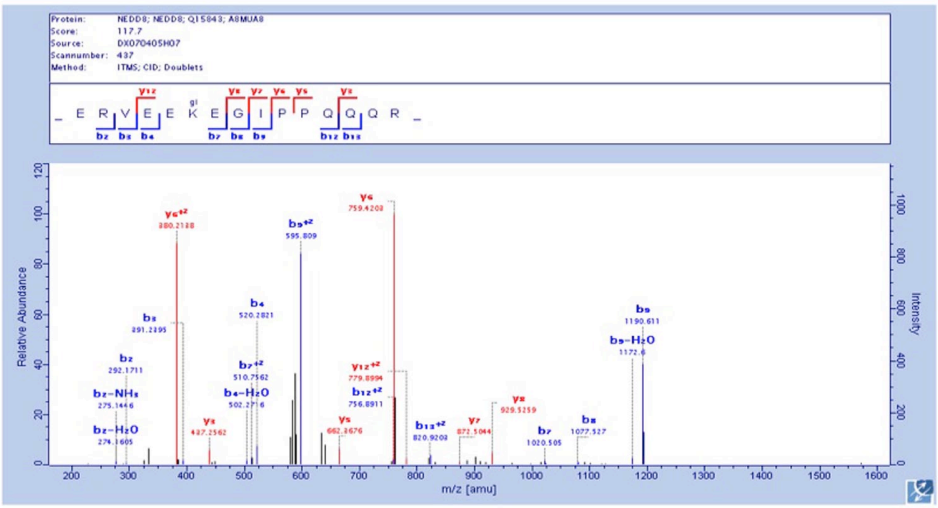
a


Protein: NEDD8; NEDD8; Q15843; A8MUA8
Score: 299.69
Source: DX070405H07
Scannumber: 1220
Method: ITMS; CID; Doublets
_ E I E I D I E P T D gl K V E R _
Relative Abundance
Intensity [10e4]
m/z [amu]

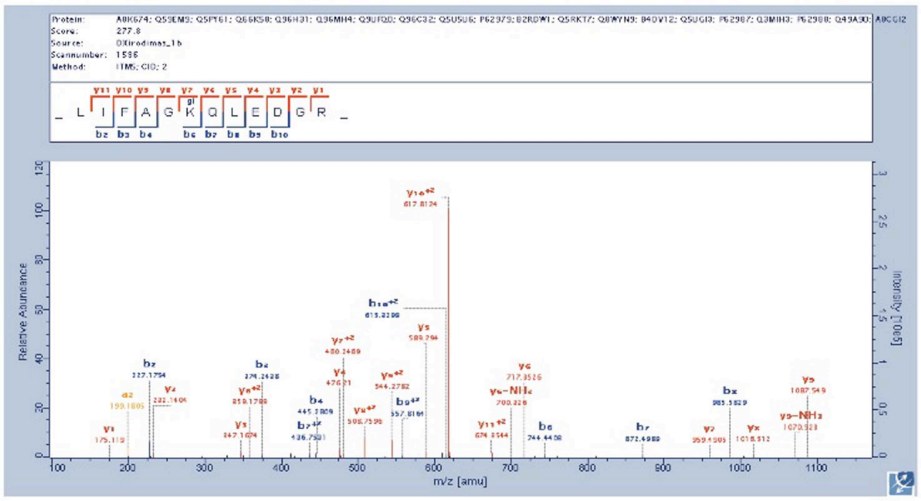
b


Protein: NEDD8; NEDD8; Q15843; A8MUA8
Score: 107.53
Source: DX070405H06B
Scannumber: 415
Method: ITMS; CID; Doublets
_ V E E K E G I P P Q Q Q R _
Relative Abundance
Intensity
m/z [amu]

c


Protein: NEDD8; NEDD8; Q15843; A8MUA8
Score: 117.7
Source: DX070405H07
Scannumber: 437
Method: ITMS; CID; Doublets
_ E R V E E K E G I P P Q Q Q R _
Relative Abundance
Intensity
m/z [amu]

d



e

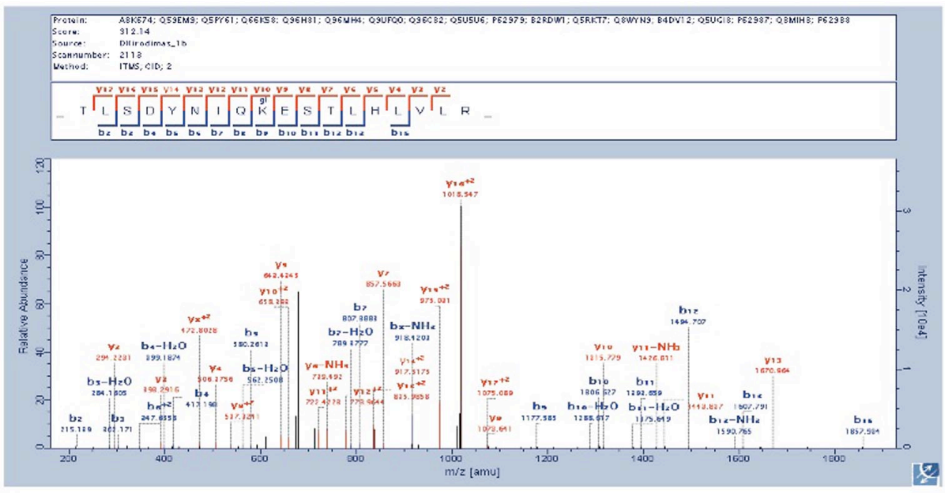


Figure 4.4: MS/MS spectra of the modified NEDD8 peptides identified in the SILAC experiment.

(a) NEDD8 peptide modified by Gly-Gly on K22. **(b)** NEDD8 peptide modified by Gly-Gly on K33. **(c)** NEDD8 peptide modified by Gly-Gly on K33 (longer peptide, due to a trypsin 'missed cleavage'). **(d)** Ubiquitin peptide modified by Gly-Gly on K48. **(e)** ubiquitin peptide modified by Gly-Gly on K63.

The screen shots also contain information on the protein, the score and the method used. Scores: (a) 299.69; (b) 107.53; (c) 117.7; (d) 277.8; (e) 912.14.

4.2.3. Quantification of the unconjugated NEDD8

Trypsin digestion of proteins occurs at the carboxyl side of lysine and arginine residues that are not modified or followed by proline. A common phenomenon during digestion is a ’missed cleavage’, which in the case of NEDD8 allows the identification of the extreme C-terminus NEDD8 peptide that indicates the presence of unconjugated free NEDD8 (Figure 4.5. a). The sequence of this peptide is not normally identified, since the common databases contain the sequence of the unprocessed form of NEDD8. The sequence of the matured form was subsequently included in the database to allow detection and quantitation of the last peptide, which is the direct evidence for the presence of unconjugated NEDD8. Our analysis identified this peptide, but interestingly MG132 treatment had no effect on its relative abundance (Figure 4.5.b, c). This observation is consistent with our western blot analysis (Figure 3.2) showing that, contrary to ubiquitin, free NEDD8 depletion does not occur when NEDD8 chains accumulate. The combination of the two analysis supports the idea that inhibition of the proteasome, while it increases the abundance of NEDD8 chains has no effect on the levels of the unconjugated NEDD8.

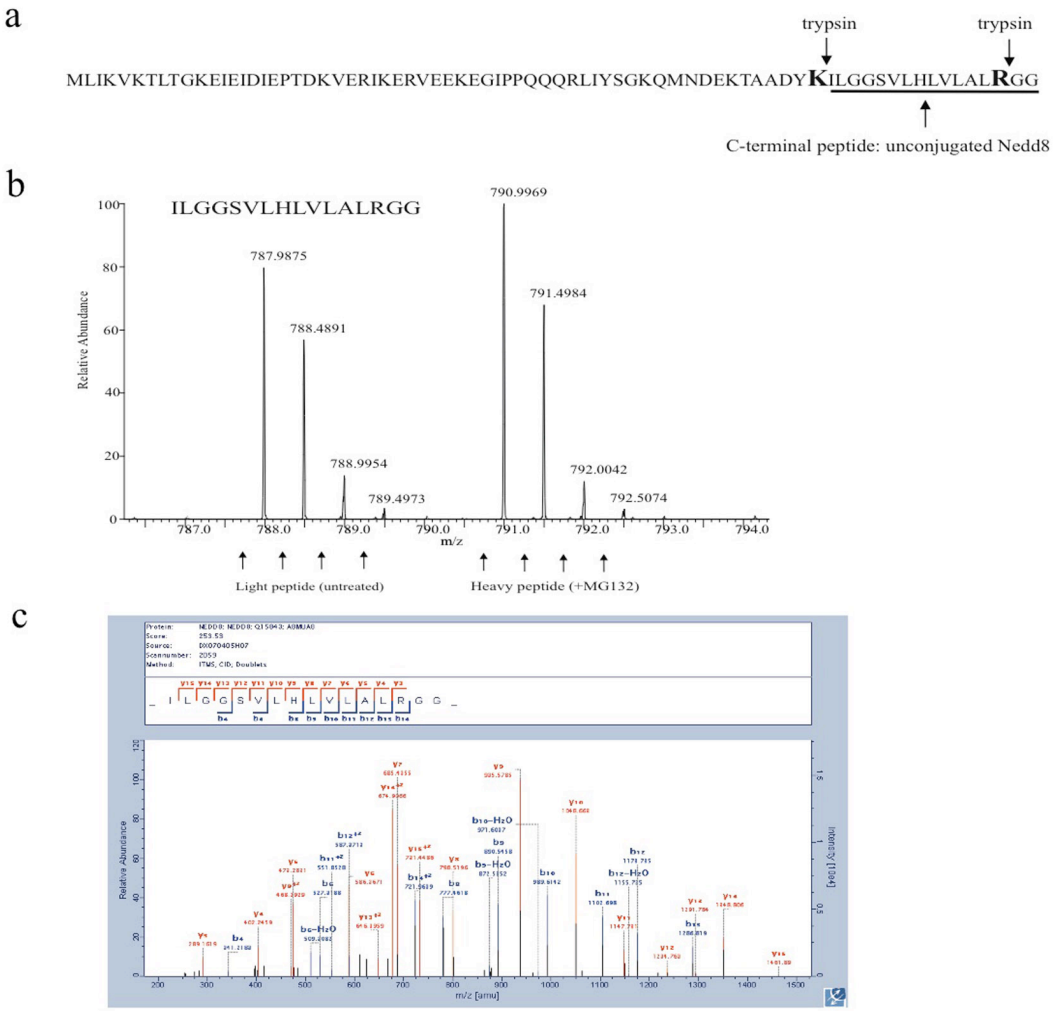


Figure 4.5: Quantification of unconjugated NEDD8.

(a) Sequence of NEDD8. Trypsin 'missed cleavage' at K60 position (no cleavage at R74) results in a unique peptide for NEDD8 (underlined). The unconjugated diglycine at the C-terminus indicates the peptide derives from unconjugated, "free" NEDD8. **(b)** MS spectrum of the last peptide of NEDD8: the SILAC ratio indicates that the relative abundance of the free form of NEDD8 is unchanged with MG132. **(c)** MS/MS spectrum of the last peptide of NEDD8.

4.2.4. Identification of ubiquitin modification on NEDD8 peptides

While the conventional strategy for the identification of diglycine motifs on lysine residues shows that NEDD8 forms chains *in vivo,* that increase upon MG132, it does not indicate the type of modification. This is because both NEDD8 and ubiquitin provide a diglycine signature upon trypsin digestion. However, it has been noted that due to ’missed clevage’, trypsin digestion produces the tetrapeptide LRGG as a modification signature on lysines[291]. Since ’missed cleavage’ in NEDD8 does not produce the LRGG signature, we used this approach to distinguish between NEDD8 and ubiquitin modification (Figure 4.6.a). We isolated NEDDylated proteins from MCF7 His_6-NEDD8 cells after MG132 treatment. The sample was digested „in solution” with trypsin for a short time, in order to achieve a partial digestion. LC-MS/MS analysis was performed on a LTQ-Orbitrap Velos mass analyzer. We added ’LRGG’ in the Andromeda search engine[250], as a posttranslational modification on lysine residues, and specifically searched for NEDD8 or ubiquitin peptides with this modification. We identified both NEDD8 and ubiquitin peptides with the LRGG signature on K48 (Figure 4.6. b-e). This finding provides the first *in vivo* evidence of ubiquitin modification on NEDD8.

a

	Tryptic digestion	Partial tryptic digestion
Ubiquitin modification on a ubiquitin peptide on K48 residue	G G LIFAGKQLEDGR	L R G G LIFAGKQLEDGR
Ubiquitin modification on a NEDD8 peptide on K48 residue	G G LIYSGKQMNDEK	L R G G LIYSGKQMNDEK

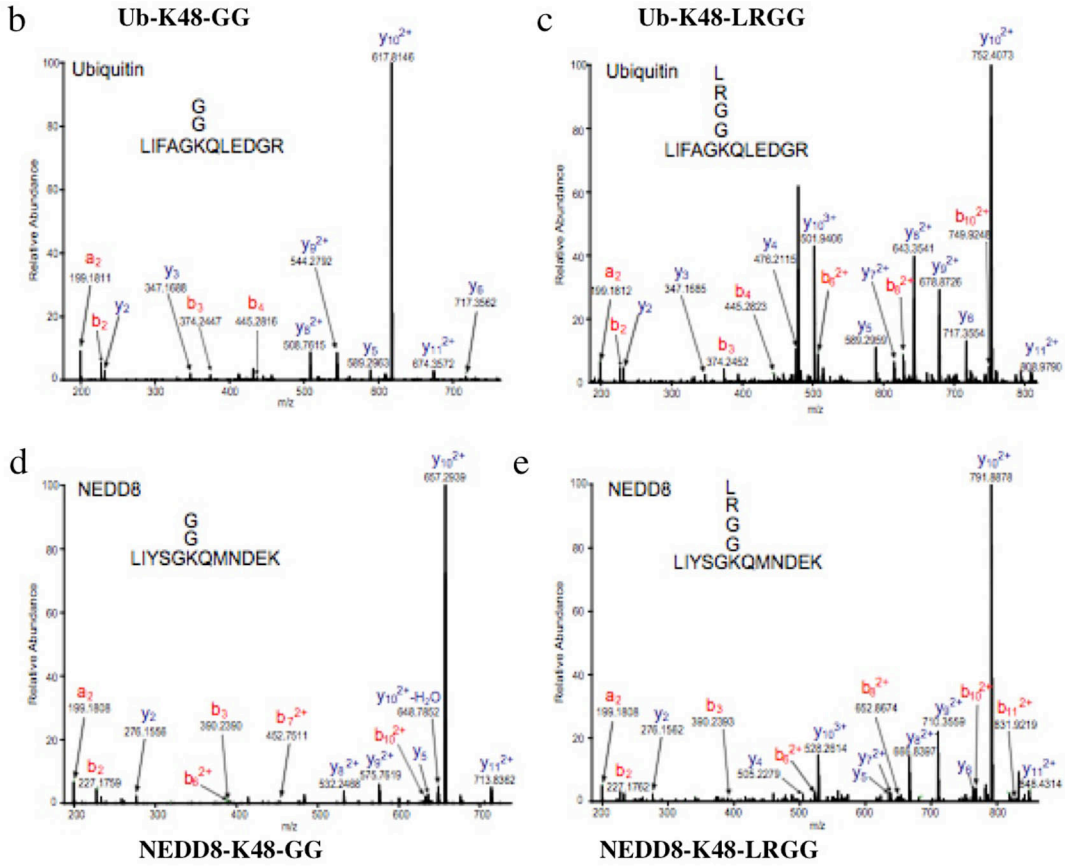


Figure 4.6: NEDD8 is modified with ubiquitin *in vivo.*

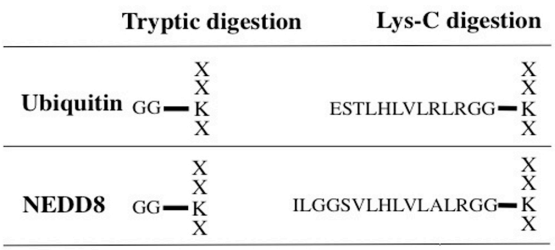
(a) Sequence of the branched peptides of ubiquitin and NEDD8 when modified by ubiquitin on K48 residue upon complete or partial trypsin digestion. **(b)** MS/MS spectrum of the ubiquitin peptide modified by GG on K48 (m/z 487.60005; (3+); mass error 0.53 ppm). **(c)** MS/MS spectrum of the ubiquitin peptide modified by LRGG on

K48 (m/z 577.32844; (3+); mass error 0.1 ppm). **(d)** MS/MS spectrum of the NEDD8 peptide modified by GG on K48 (m/z 513.92057; (3+); mass error 0.5 ppm). **(e)** MS/MS spectrum of the NEDD8 peptide modified by LRGG on K48 (m/z603.64897; (3+); mass error 0.4 ppm).

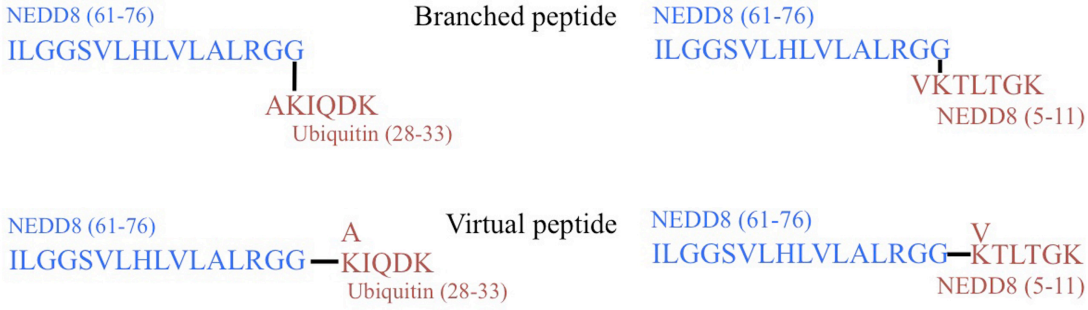
4.2.5. Identification of NEDD8-NEDD8 and NEDD8-ubiquitin branched peptides with a different strategy

Although successful with ubiqutin, the above described strategy did not allow the identification of peptides modified by NEDD8. Therefore, we turned to another approach to distinguish between NEDD8 and ubiquitin modification: instead of trypsin digestion, NEDDylated proteins isolated from MCF7-ND8 cells after MG132 treatment were digested in solution with endoproteinase Lys-C. This enzyme provides unique signatures for either NEDD8 or ubiquitin modification on lysine residues, since it cleaves at the C-terminus of lysine, but not arginine residues (Figure 4.7. a)[292]. We created a new database that included the predicted NEDD8 and ubiquitin branched peptides, and processed our data obtained from the mass spectrometric analysis for the identification of ubiquitin and NEDD8 branched peptides (Figure 4.7. b, c [293]). We found that ubiquitin is NEDDylated on K29 and NEDD8 is NEDDylated on K6 lysine residue (Figure 4.8. a, b). The combination of the two approaches (partial tryptic and Lys-C digestion) suggests that in cells NEDD8 forms chains with NEDD8 and ubiquitin as well. The detection and analysis of branched peptides in complex mixtures is technically very challenging [294]. Thus, based on the previous identification of multiple modification sites on both NEDD8 and ubiquitin[106, 107] it is highly possible that the identified types of modification for K48 and K6 in NEDD8 and K29 in ubiquitin will apply to other NEDD8/ubiquitin sites. Nevertheless, our data for the first time indicate the formation of mixed NEDD8-ubiquitin chains *in vivo*.

a



b



c

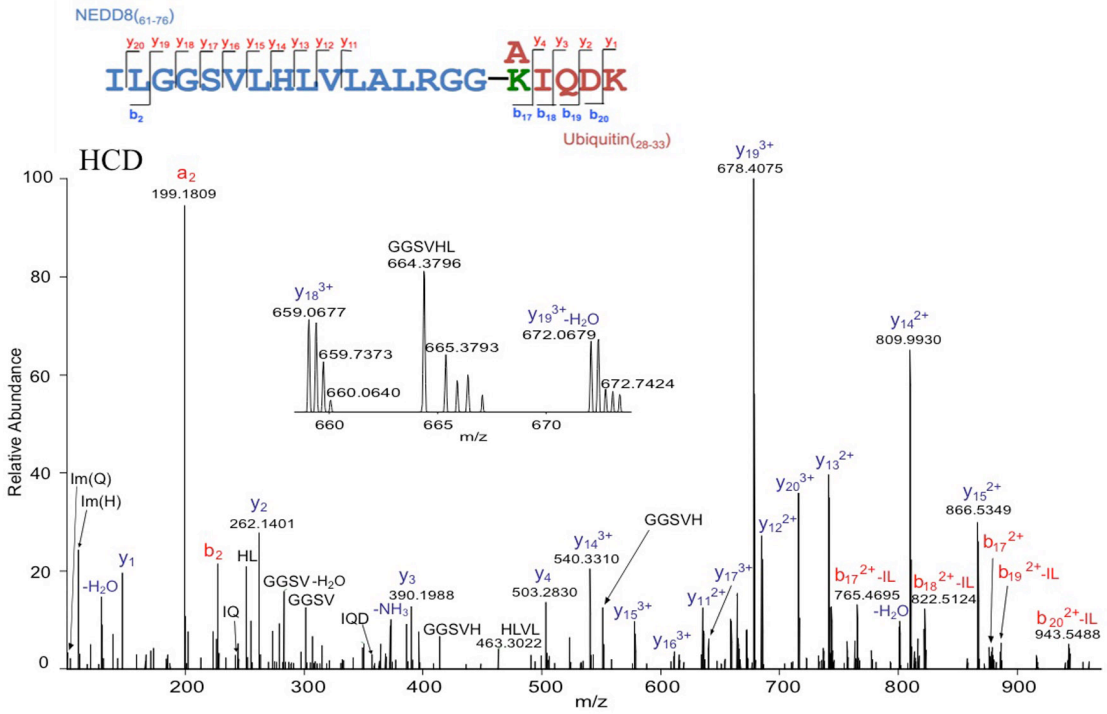
>IPI:NEDD8-K6-NEDD8|XX|XX|NEDD8-K6-NEDD8.
ILGGSVLHLVLALRGGVKTLTGK

>IPI:NEDD8-K29-ubiquitin|XX|XX|NEDD8-K29-ubiquitin.
ILGGSVLHLVLALRGGAKIQDK

Figure 4.7: Branched peptide sequences

(a) Sequence of the branched peptides of ubiquitin and NEDD8 on lysine residues upon trypsin or Lys-C digestion. While complete trypsin digestion leaves a diglycine signature on lysines, Lys-C digestion enables discrimination between the different modifiers. (X: any amino acid) **(b)** Sequence of the NEDD8-ubiquitin and NEDD8-NEDD8 branched peptides and the virtual peptides. For the easy interpretation of the MS/MS spectrum, the cross-linked peptide is "reversed"[294]. **(c)** The two sequences show examples of the linearized version of the branched peptides added to database[293].

a


NEDD8(61-76)
ILGGSVLHLVLALRGG
Ubiquitin(28-33)
AKIQDK
HCD
Relative Abundance
m/z
a2 199.1809
y2 262.1401
y1
-H2O
Im(Q)
Im(H)
b2
HL
IQ
GGSV-H2O
GGSV
IQD
y3 390.1988
-NH3
GGSVH
HLVL 463.3022
y4 503.2830
y14 3+ 540.3310
GGSVH
y15 3+
y16 3+
y11 2+
y17 3+
y12 2+
y20 3+
y13 2+
y19 3+ 678.4075
y14 2+ 809.9930
y15 2+ 866.5349
b17 2+
b19 2+-IL
b18 2+-IL 822.5124
b17 2+-IL 765.4695
-H2O
b20 2+-IL 943.5488
y18 3+ 659.0677
659.7373
660.0640
GGSVHL 664.3796
665.3793
y19 3+-H2O 672.0679
672.7424

b

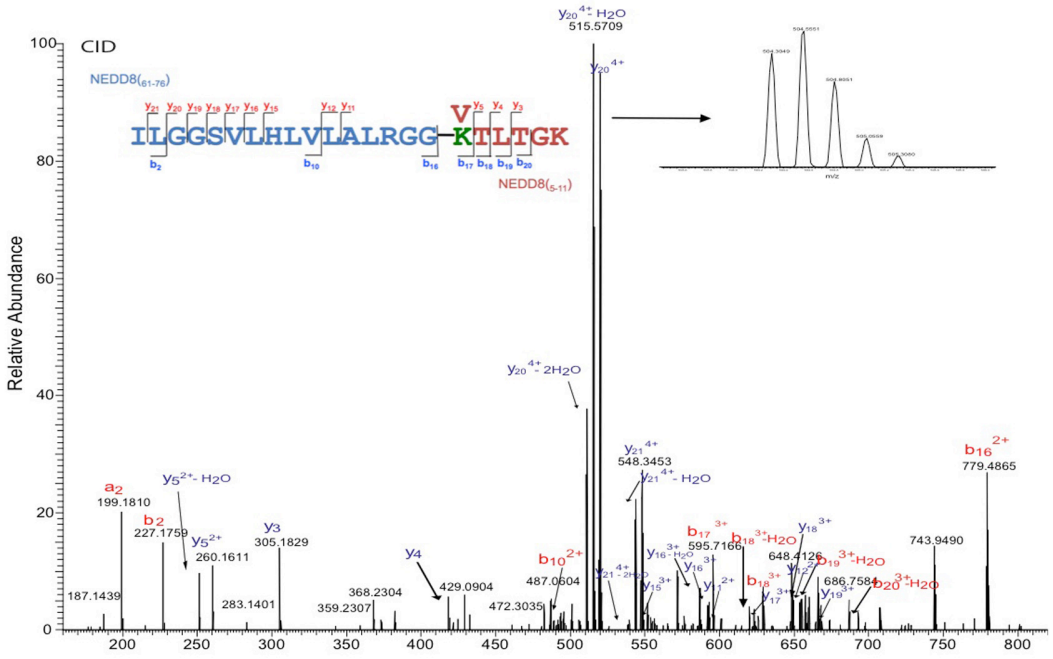


Figure 4.8: NEDD8 modifies ubiquitin on Lys29 and NEDD8 on Lys6

MCF7-ND8 cells were treated with 30 μM MG132 for 4 hrs. Isolated NEDDylated proteins were digested with Lys-C to enable discrimination between NEDD8 and ubiquitin sites. After MS analysis, peptides were identified using MaxQuant software and a modified database containing Lys-C digested NEDD8 and ubiquitin branched peptides. Figure **(a)** represents the high-resolution MS/MS spectrum of a ubiquitin peptide modified with NEDD8 on K29 (m/z 452.47996 (5+); mass error -0.3 ppm). The peptide was fragmented by high-energy collision-induced dissociation (HCD) and the fragment ions were analysed in the orbitrap analyser. Figure **(b)** represents the high-resolution MS/MS spectrum of a NEDD8 peptide modified with NEDD8 on K6 (m/z 461.29248 (5+); mass error 0.1 ppm). The peptide was fragmented by collision-induced dissociation and the fragment ions were analysed in the orbitrap analyser. Spectra were assigned manually.

4.2.6. NEDD8 is present on ubiquitinated substrates recognised by ubiquitin-binding domains that are associated with the 26S proteasome

The signals created by the different types of ubiquitin chains are decoded by the recognition of ubiquitin-binding domains (UBDs). In order to gain insight into the role of Ube1-mediated NEDD8 activation, we investigated how different UBDs or UBD containing proteins recognize NEDD8/mixed chains. We obtained constructs of GST-tagged Rpn10, Rpn13, and the UBA domains of Rad23A, B, and Plic2 from Dr. Koraljka Husnjak. These proteins are either part of the 26S proteasome (Rpn10 and Rpn13) or play role in recognizing ubiquitinated proteins and transferring them to the proteolytic machinery (Plic2, Rad23 and Sc. Dsk2). We expressed them as recombinant GST fusion proteins in bacteria (Figure 4.9) and used them in pull down assays with untreated, MG132, or heat shock treated H1299 cell extracts. Cells were lysed and incubated overnight with the indicated recombinant GST-UBA-domain that was coupled to GSH-activated sepharose beads. After washes, conjugates were eluted with 2X SDS and analyzed by western blotting with NEDD8 and ubiquitin antibodies (Figure 4.10. a, b). After detection, membranes were washed and stained with coomassie blue reagent to visualize the total proteins isolated with the UBDs (Figure 4.10. c). Our preliminary data show that these UBDs /proteins pull down NEDD8/mixed chains when cells are treated with MG132 or heat shock, indicating that the chains recognized by these proteins contain NEDD8.

Under unstressed conditions, a band corresponding to NEDDylated cullins appears from the pull-downs with all tested receptors, but not when only GST was used as a control. From this experiment it cannot be concluded whether the UBDs recognize cullins or the NEDD8 modification. The SCF ligase complex has been co-purified with the 19S cap of the proteasome[295]; therefore direct physical interaction between cullins and

ubiquitin receptors is not surprising. It is possible that cullins come as part of the SCF complex including the ubiquitinated substrate. On the other hand, it is also likely that NEDD8 is recognised by the receptors. NEDD8 shares similar three-dimensional structure (the β-fold) with ubiquitin, and possesses the Ile44 patch (Leu8, Ile44, Val70) as well, which is the platform of recognition for the majority of UBD-containing proteins[74].

Besides cullins, other NEDDylated proteins have also been detected from the input under untreated conditions; while in the pulldowns, only cullins (the most abundant NEDD8-conjugates) and a band at ~37 kD are observed most of the time (this is probably due to the secondary antibody recognizing the recombinant proteins). An interesting exception is the Rpn10 pulldown, where a few other low molecular weight bands were detected. These proteins are most likely targeted for degradation under normal conditions, and recognized by Rpn10 on the 26S proteasome.

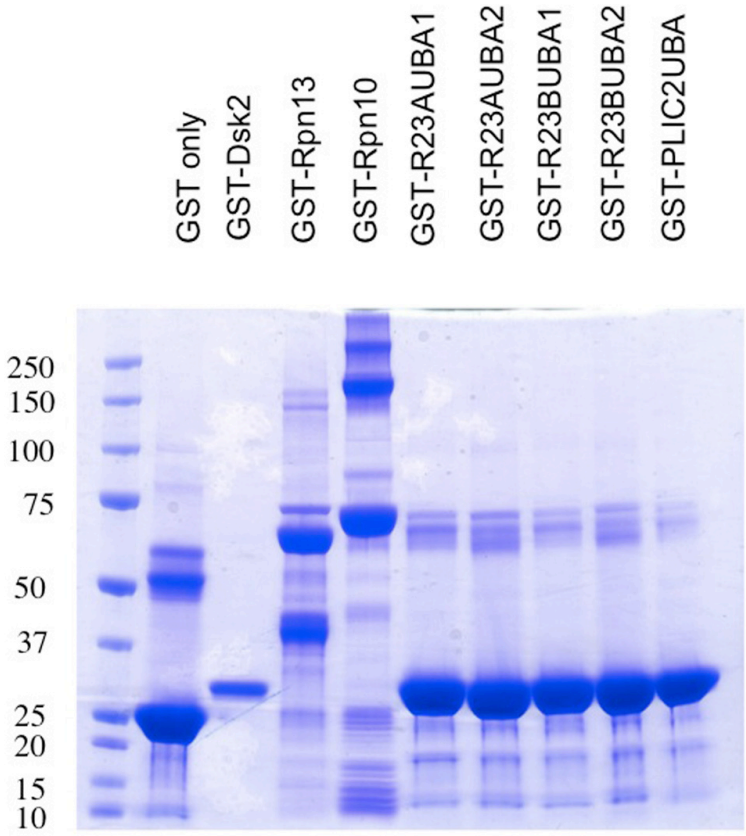
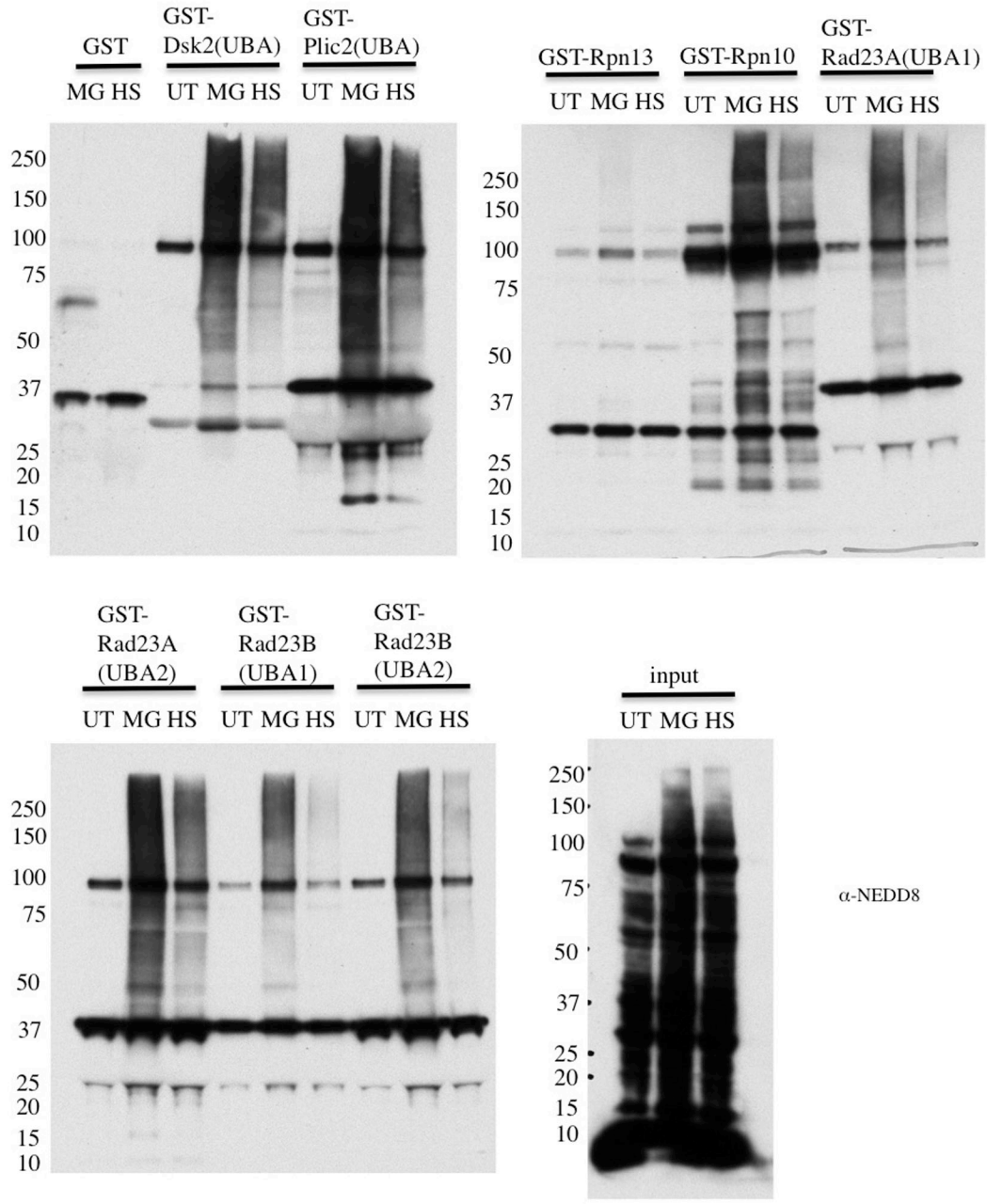


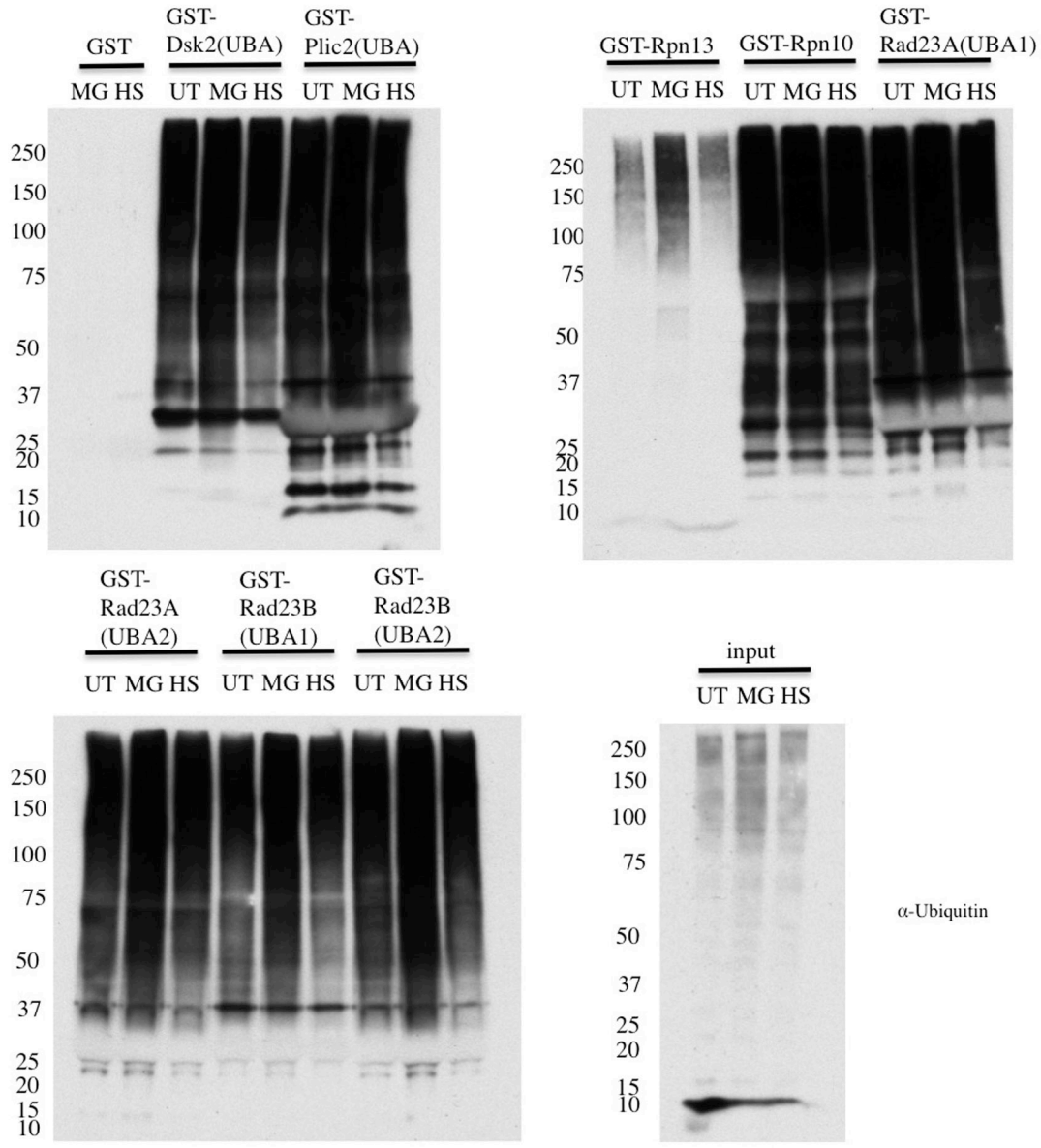
Figure 4.9: Expression of the UBA domains or UBD-containing proteins

GST-tagged UBDs were expressed in BL21-CodonPlus® Competent Cells and purified as discussed in the Materials and Methods. Recombinant proteins were stored coupled to beads. To monitor the expression of the proteins, equal amounts of beads were boiled in 2XSDS, loaded on SDS-PAGE and stained with Coomassie blue reagent.

a


GST
GST-Dsk2(UBA)
GST-Plic2(UBA)
MG HS
UT MG HS
UT MG HS
250
150
100
75
50
37
25
20
15
10
GST-Rpn13
GST-Rpn10
GST-Rad23A(UBA1)
UT MG HS
UT MG HS
UT MG HS
GST-Rad23A (UBA2)
GST-Rad23B (UBA1)
GST-Rad23B (UBA2)
UT MG HS
UT MG HS
UT MG HS
input
UT MG HS
α-NEDD8

b


GST
GST-Dsk2(UBA)
GST-Plic2(UBA)
MG HS UT MG HS UT MG HS
250
150
100
75
50
37
25
20
15
10
GST-Rpn13
GST-Rpn10
GST-Rad23A(UBA1)
UT MG HS UT MG HS UT MG HS
GST-Rad23A (UBA2)
GST-Rad23B (UBA1)
GST-Rad23B (UBA2)
UT MG HS UT MG HS UT MG HS
input
UT MG HS
α-Ubiquitin

c

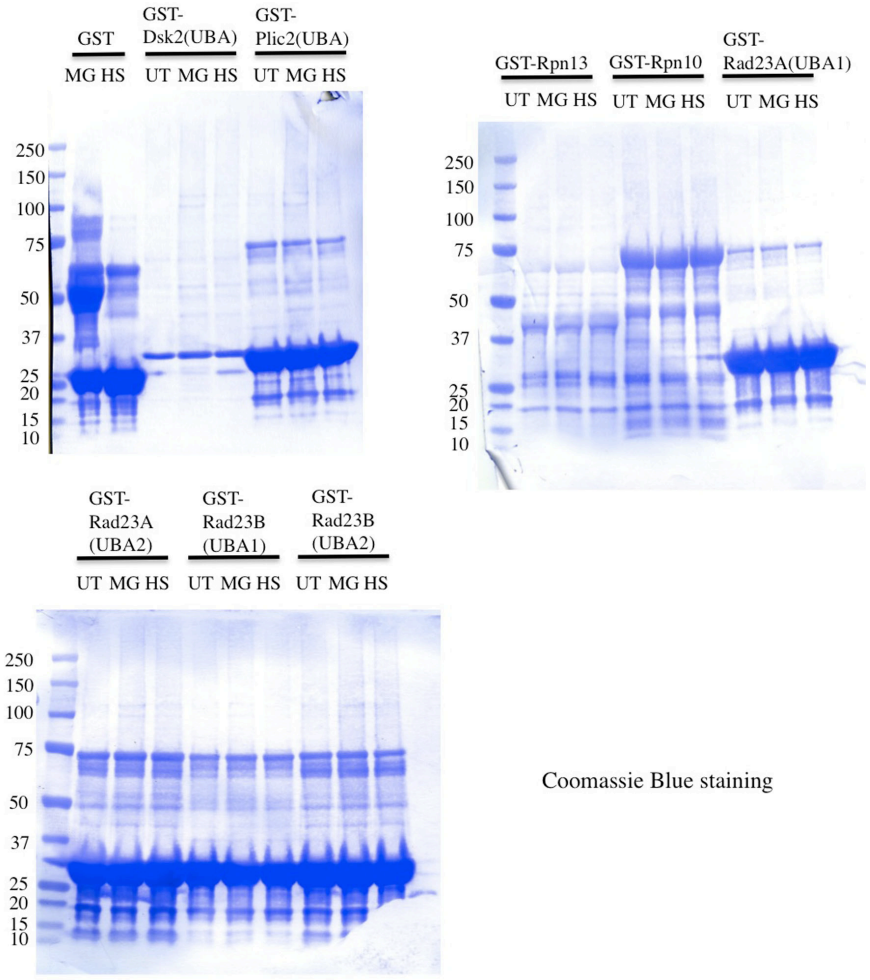


Figure 4.10: NEDD8 is present in the ubiquitin chains recognised by ubiquitin-binding proteins associated with the ubiquitin-proteasome system.

H1299 Cells were either treated with 30 μM MG132 for 4 hrs, or heat shocked at a 43^{o}C incubator for 1 hour, or left untreated, as indicated. Lysates were incubated with GST-UBA-domains or full-length proteins coupled to Glutathione Sepharose beads overnight. The eluates were mixed with 2X SDS and analysed by western blotting with **(a)** NEDD8- or **(b)** ubiquitin-specific antibodies. **(c)** After detection, membranes were stained with coomassie blue reagents to visualize the total proteins isolated in the pulldowns.

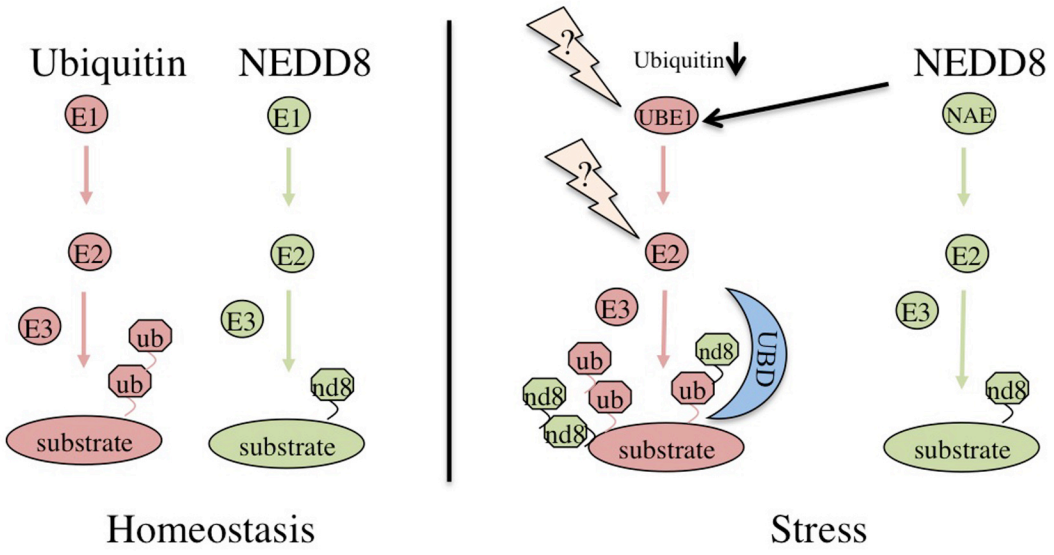


Figure 4.11: Schematic representation of the ubiquitin and NEDD8 conjugation pathways under homeostatic and stress conditions.

4.3. Discussion and future directions:

Mass spectrometry-based proteomics is a powerful technique that provides a large amount of information on proteins and PTMs. Reprocessing of 'old' raw data with more advanced software tools and expanded databases or searching for a modification that has not been looked at before ('LRGG') in the dataset provided us with valuable new information on NEDD8 chain formation.

Diglycine modifications on NEDD8 peptides have been found earlier, indicating that NEDD8 forms chains[106, 107]. However, the modifier and the type of modification have not been addressed before. We applied two mass spectrometry based methods to unambigously identify ubiquitination and NEDDylation sites. Our data provide the first evidence on the formation of di-NEDD8 (which most likely indicates poly-NEDD8) and mixed NEDD8-ubiquitin chains *in vivo*. As already discussed, while our analysis identified K48 and K6 in NEDD8 as ubiquitination and NEDDylation sites respectively, and K29 on ubiquitin as NEDDylation site, it is very likely that this type of modification applies to other NEDD8 and ubiquitin lysines.

Despite this success, these methods are inadequate for the detection of sites on substrate proteins. This is due to a) the relative low abundance of the NEDDylated peptides and b) difficulties in interpreting the resulting MS/MS spectra[294]. Another problem is that standard databases do not contain the branched peptides that derive from Lys-C digestion; therefore the identification is only possible if a new database is created with all the potentially modified substrates. Jeram et al. have developed a database search software, SUMmOn for identification of ub/ubl sites after Lys-C digestion[296]. They have successfully identified NEDDylation sites *in vitro*. However, the methodology has not been used *in vivo*.

There are other ways to improve large-scale NEDD8 site identification; I am only going to discuss one in this thesis. As mentioned above, ubiquitin and NEDD8 both leave the diglycine motif on proteins due to the tryptic cleavage after the arginine at position 74. To overcome this problem, one could introduce an arginine74 to lysine mutation (R74K) on NEDD8, and apply Lys-C digestion to obtain the diglycine signature only on substrates that are modified with the R74K mutant of NEDD8 (see on Figure 4.12). One could use then the recently developed monoclonal antibody that recognizes the diglycine motif to enrich for peptides that contain NEDDylation sites. This is only one of the several ways NEDD8 site identification can be and will be improved in the following years.

Figure 4.12: Sequence of NEDD8 and ubiquitin with the C-terminal Lys-C cleavage sites.

Large-scale NEDD8 site identification with easily applicable methods will answer important biological questions, such as whether NEDD8 modifies proteins directly on their acceptor lysine residue, or through intercalation into ubiquitin chains formed prior to NEDDylation.

Recently a group has also shown evidence on NEDD8 modification on ubiquitin, however they did not detect ubiquitin modification on NEDD8 or NEDD8 chains, and concluded that NEDD8 might act as a chain terminator (Michael Glickman, oral presentation at the ZOMES VII meeting). We have identified ubiquitin and NEDD8 modification on NEDD8 by mass spectrometry, suggesting that NEDD8 intercalates into chains, instead of terminating them. However, our experiment was done in the presence of MG132 that induces a vast Ube1-mediated NEDDylation. Under physiological conditions such strong trigger probably does not occur, and milder NEDD8 engagement into the ubiquitin pathway might result in only a single NEDD8 modification on ubiquitin chains, similarly what was detected in *S. cereviseae*. In this case, NEDD8 might indeed play role in chain termination, which could allow the recovery of the free ubiquitin pools that are depleted upon stress. Therefore, it would be interesting to test the type of branched peptides that one can detect from heat shock treated cells, which results in a milder (and more physiological) effect on NEDD8 activation.

Some groups have also detected unanchored NEDD8-ubiquitin chains in *S. cereviseae* (Thimo Kurz, personal discussion and Michael Glickman, oral presentation at the ZOMES VII meeting). Whether NEDD8 modifies substrates through the ubiquitin pathway or this type of NEDDylation results in accumulation of unanchored chains (or both possibilities exist at the same time) need to be further addressed. There have been several reports on free polyubiquitin chains. They are generated during ubiquitin biosynthesis, by DUBs that release chains from the substrates or they can be synthetized by ubiquitinating enzymes[297]. Unanchored Lys63 polymers have been shown to be important for signal transduction, acting as transient platform for protein interactions[298]. It would be also important to determine the biological outcome of the unanchored mixed chain formation, such as their recognition by cellular receptors. It is

likely that UBD-containing proteins bind to them. NEDD8 intercalation to free chains might provide a different platform for biological processes, or alter half-life of the chains that are normally quickly disassembled by DUBs.

The research on ubiquitin protein modification has revealed an exquisite and diverse functional output that depends on the type of chain formation of the same modifier (K48, K63, K11, linear ubiquitin chains) [37, 299]. Therefore, it is very likely that the identified types of NEDD8 modification under stress conditions create a new signal (poly-NEDD8 chains) and/or alter the biological outcome of the ubiquitin signal.

The well-established role for NEDD8 is the indirect regulation of protein ubiquitination through modification of cullins and the control of CRL activity. Our data now provide evidence for a direct role of NEDD8 in controlling the ubiquitin signal output, as the NEDD8 conjugates are also ubiquitinated through formation of mixed chains. Therefore, based on this mechanism and on the identification of K48 both in NEDD8 and ubiquitin as a chain formation site, and the mixed chain interaction with the UPS, a possible role for NEDD8 could be the control of the rates of transport, recognition and/or degradation of ubiquitinated proteins. However, as the biological output of ubiquitin modification is extended well beyond protein degradation, additional regulatory functions for NEDD8 may exist.

We have used recombinant UBA domains and full-length proteins in order to uncover whether the mixed chains signal to the UPS. All of the tested proteins/domains pulled down NEDD8 conjugates to different extend. From this assay, many conclusions can be drawn and even more questions can be raised that I am going to discuss below. When interpreting data from such pulldown experiments, it is important to keep in mind that isolated domains might behave differently to the full-length proteins. Therefore the data obtained is interpreted as a preliminary observation that has to be further investigated.

Whether or not the domains can bind directly to NEDD8 or the interaction is through ubiquitin remains to be determined. It has been shown that UIMs (ubiquitin interacting motifs) of certain proteins recognize both NEDD8 and ubiquitin[103]. Interestingly, in yeast two-hybrid systems 30% of Ubiquitin Binding Domains (UBDs) appeared to bind NEDD8 as well [300]. In order to gain insight into the nature of the recognition, further biochemical studies have to be performed. One possible way is to create short NEDD8 chains or mixed chains and screen them in yeast two-hybrid assays with a library containing UBA-domains. This is going to be investigated in Koraljka Husnjak's lab in the future. Another possibility is to measure the affinity of UBD-containing proteins toward recombinant NEDD8/mixed chains, and compare them to ubiquitin chains of different linkage. It would be interesting to find out whether there are proteins that have higher specificity towards NEDD8, than ubiquitin, or whether receptors' affinity towards ubiquitin chains is altered when NEDD8 is intercalated? These possibilities suggest that recognition and processing of the mixed chains by the 26S proteasome would be altered, and would also probably affect signaling involved in other cellular processes. In this thesis, we only investigated NEDD8 binding to proteasome receptors. Since NEDD8 is involved in a variety of cellular processes, it would be interesting to test the affinity of other UBD-containing proteins, such as the ones involved in DNA damage, towards this ubl.

I find it highly relevant to mention some of the new observations that other groups made on the same subject. Michael Glickman has shown their new finding on NEDD8-ubiquitin chains and their recognition by proteasome receptors and shuttle proteins at the ZOMES VII meeting on "Ubiquitin family proteins and their cognate PCI complexes" in September, 2012. First of all, they have obtained the structure of ScRub1 (NEDD8) by NMR, which appeared to be fully superimposable to that of the ubiquitin. The conserved Ile44 patch is identically positioned to the ubiquitin patch, suggesting

direct recognition by UBA-domains. Next, they mapped binding of domains to Rub1 by NMR CSR; and found that Rad23 and Dsk2 recognise Rub1 very similarly to ubiquitin. However, Rpn10, the ubiquitin receptor on the 26S proteasome showed higher affinity towards ubiquitin.

Adaptors for NEDD8 have also been identified, such as the NEDD8 interacting protein NUB1 (NEDD8 Ultimate Buster 1) that is suggested to play role in recruiting NEDDylated substrates and NEDD8 monomer to the 26S proteasome for degradation[115, 116]. An alternative explanation for the accumulation of NEDDylated substrates upon stress is that they are normally targeted to the proteasome, possibly through modification of NEDD8 with K48 ubiquitin chains. The observation that no significant depletion of free NEDD8 is detected upon stress while NEDD8 conjugation is dramatically increased, is consistent with the idea that NEDD8 itself may be a substrate for proteasomal degradation.

Another big question, apart from how NEDD8 is recognised by the proteasome, is whether and how DUBs on the degradation machinery can recognise and/or process the NEDD8/ubiquitin mixed chains. If they do so, is NEDD8 removal as efficient as ubiquitin? There have been three proteins identified on the proteasome that possess DUB activity: Rpn11, Uch37 and Usp14. None of them has been shown to have deNEDDylating activity. The COP9 signalosome, the protein complex dedicated for deNEDDylation has been suggested to be transiently associated with the proteasome, or even is able to replace the lid of the degradation machinery[133, 134]. These findings however are debated in the field.

There have been ~ 90 DUBs identified so far that are not associated with the 26S proteasome and are involved in diverse cellular processes. Some of the ubiquitin C-terminal hydrolases (UCHs) show dual specificity towards NEDD8 and ubiquitin

(UCH-L3, UCH-L1, PfUCH54). In *in vitro* studies, ovarian tumor-related proteases (OTUs) appear to bind to NEDD8, but do not possess deNEDDylating activity (personal discussion with David Komander). Among the large class of ubiquitin-specific proteases (USPs), only Usp21 has been reported to have NEDD8 cross-reactivity[301]. However, a later study has not detected NEDD8 cleavage in the presence of Usp21[114]. Moreover, based on structural studies, the authors determined amino acids on NEDD8 and USPs that prevent NEDD8 from being targeted by these types of proteases. Interaction between ubiquitin Arg72 residue and the USP domains is essential for processing ubiquitin. NEDD8 possesses an alanine residue at the 72 position, therefore USPs do not have affinity toward this ubl. Interestingly, amino acids on the N-terminus of NEDD8 (that are localized on the β1 and β2 strands), namely Lys4, Glu12 and Glu14 also preclude USP binding, due to steric clashes and charge repulsion[114].

Previous studies[102, 103, 260, 261] have shown that many ubiquitin E2s can form thiolester bond with NEDD8 *in vitro* and shared E3 ligases between NEDD8 and ubiquitin exist. It is becoming evident that most of the ubiquitin E2 and E3 enzymes do not discriminate between the two posttranslational modifiers. However, a large portion of deubiquitinating enzymes appears to have specificity towards ubiquitin. NEDD8 intercalation into the polyubiquitin signal might inhibit the isopeptidase activity of DUBs that cannot cleave NEDD8 from mixed chains. The biological relevance of the NEDD8 mixed chain formation can be addressed by testing how these chains are processed by DUBs that are involved in important regulatory mechanisms and what is the consequence of the supposititious inhibition in DUB activity by NEDD8 intercalation into ubiquitin chains. Usp5 has been reported to cleave unanchored ubiquitin chains from the proximal end. It would be interesting to test whether it can disassemble the potential ubiquitin-NEDD8 unanchored chains as well.

To sum up, in this project we have characterized the chains formed by the ubiquitin conjugation cascade. We identified that NEDD8 is present in polyubiquitin signals. We have also determined that the mixed chains are recognized by ubiquitin binding domains. Based on this observation, recombinant UBDs could be used in the future to isolate NEDD8-ubiquitin mixed chains for further studies. For instance, endogenous NEDD8 conjugates could be pulled down with the recombinant protein Plic-2 after heat shock or other cellular stimuli and subjected to MS-analysis. Moreover, ubiquitin or ubiquitin/NEDD8 mixed chains could be specifically isolated and their properties could be compared in biochemical assays (e.g. presenting them to the 26S proteasome and monitoring the rates of substrate degradation). In addition, based on our preliminary data, Rpn10 appears to be a right tool to isolate NEDDylated proteins under homeostatic conditions for proteomics studies.

Chapter 5: Role of the NEDP1 deNEDDylating enzyme in DNA-damage induced apoptosis

5.1. Introduction

5.1.1. NEDP1 and its orthologs

The NEDP1 deNEDDylating enzyme is highly conserved throughout evolution (see Figure 5.1), and has been studied in multiple organisms, including *Drosophila melanogaster* (DEN1), *S. pombe* (Nep1 and Nep2), and humans (NEDP1/SENP8).

It is a cysteine protease (the catalytic cysteine being Cys163), and contains the His-Asp-Cys catalytic (triad) residues also present in sentrin-specific proteases (SENPs) and the yeast Ulp1 (Figure 5.2). Interestingly, it does not share sequence homology to ubiquitin-specific proteases, and is highly specific towards NEDD8[90, 302]. It processes neither SUMO, nor ubiquitin with the C-terminal extension. Structural studies revealed that the isopeptidase undergoes a conformational change upon NEDD8 binding. The residues on NEDD8 that are important for NEDD8-ubiquitin discrimination are engaged in catalysis when a flexible loop centred on Q96 in NEDP1 swings over the C-terminus of NEDD8, and locks it into an extended β-structure. The Ala72 residue at the C-terminus of NEDD8 is essential for recognition and processing by NEDP1. Since this residue is not conserved in ubiquitin (Arg72), this amino acid provides the molecular determinant for NEDP1's specificity towards NEDD8[90, 303].

NEDP1 is able to process NEDD8 precursor immediately after the diglycine motif, leaving the mature, conjugable NEDD8 form. It is also capable of removing NEDD8 from substrates. It has been shown to deNEDDylate cullins *in vitro* and *in vivo* in overexpression experiments, although its activity to deNEDDylate cullins under physiological conditions is debated. For example *nep1* and *nep2* (the NEDP1 homologs in *S. pombe*) deletions do not seem to affect cullin NEDDylation, while deletion of the

CSN complex clearly result in increased cullin NEDDylation. However, in the strains where the NEDP1 homologs are deleted, high-molecular weight NEDDylated species accumulate, indicating the presence of other substrates for NEDP1's deNEDDylating activity. The same phenomenon was observed in *Drosophila melanogaster*: *den1* deletion does not affect endogenous Cul1 and Cul3 NEDDylation, while other NEDDylated proteins accumulate[304]. Recently, a few more groups independently found that non-cullin NEDDylated substrates accumulate upon deletion of NEDP1 homologs in different species, such as *Arabidopsis thaliana* and *Aspergillus nidulans* (ZOMES VII meeting, personal discussions).

Overexpressed NEDP1 has been shown to deNEDDyate p53[102], full-length p73[144], L11[148], the caspase-7 ortholog in *Drosophila*, drICE[104] and more recently E2F-1[150, 151]. However, BCA3 is the only physiological target for NEDP1 that has been identified so far under entirely endogenous conditions[146].

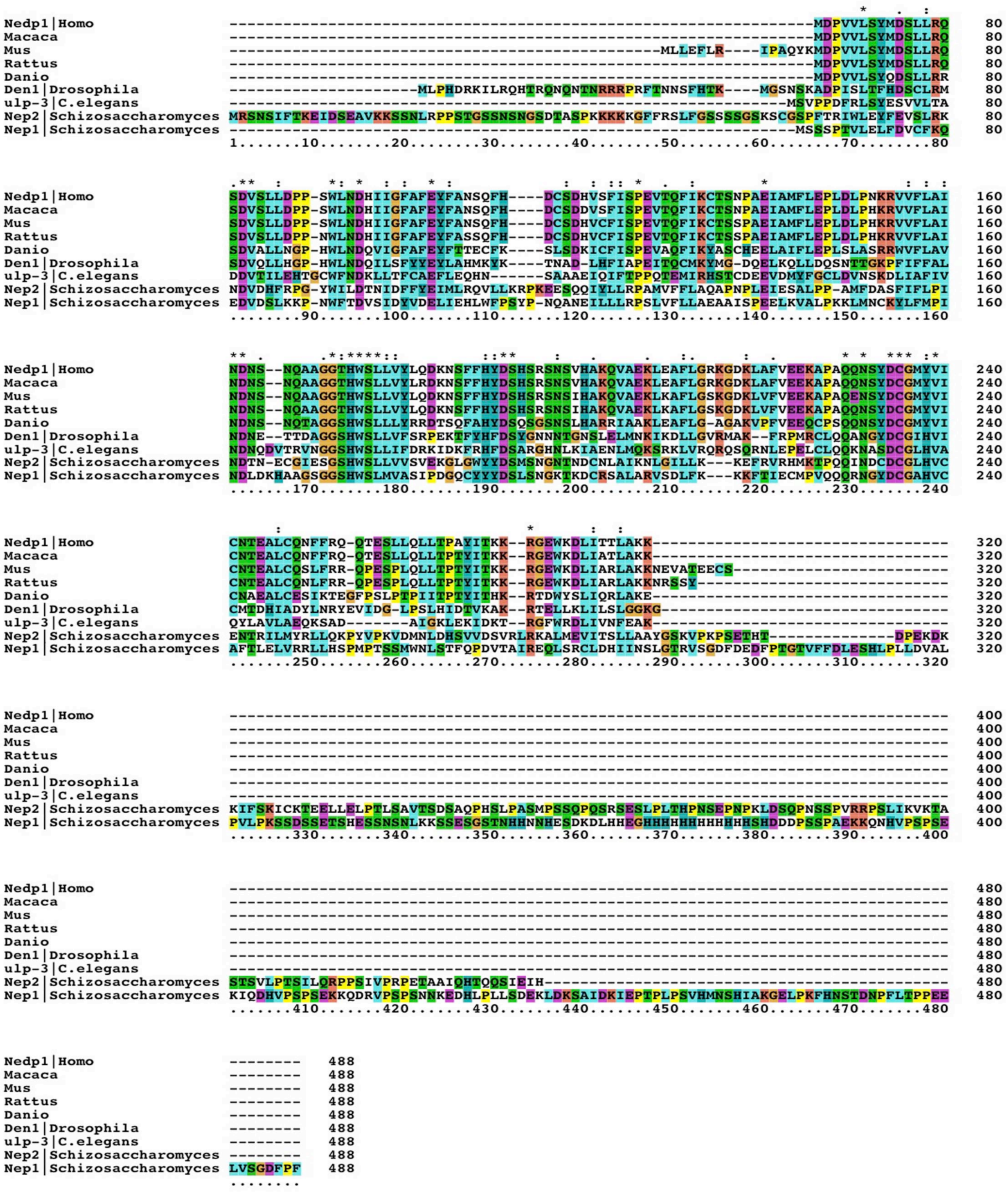


Figure 5.1: NEDP1 is highly conserved between species.

Sequence alignment between *Homo sapiens* NEDP1 (accession number: NP_001159812.1), *Macaca mulatta* (XP_001089430.1), *Mus musculus* (NP_001165542.1), *Rattus norvegicus* (NP_001012355.1), *Danio rerio* (NP_001070633.1), *Drosophila melanogaster* Den1 (NP_001163126.1), *Caenorhabditis elegans* ulp-3 (NP_001023477.1), *Schizosaccharomyces pombe* nep1 (NP_596375.2) and nep2 (NP_595608.1) was performed using ClustalX 2.0.12 software. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar

properties. A . (period) indicates conservation between groups of weakly similar properties. The residues are coloured according to their physicochemical properties (red: small + hydrophobic; blue: acidic; magenta: basic; green: hydroxyl + sulfhydryl + amine + G; grey: others).

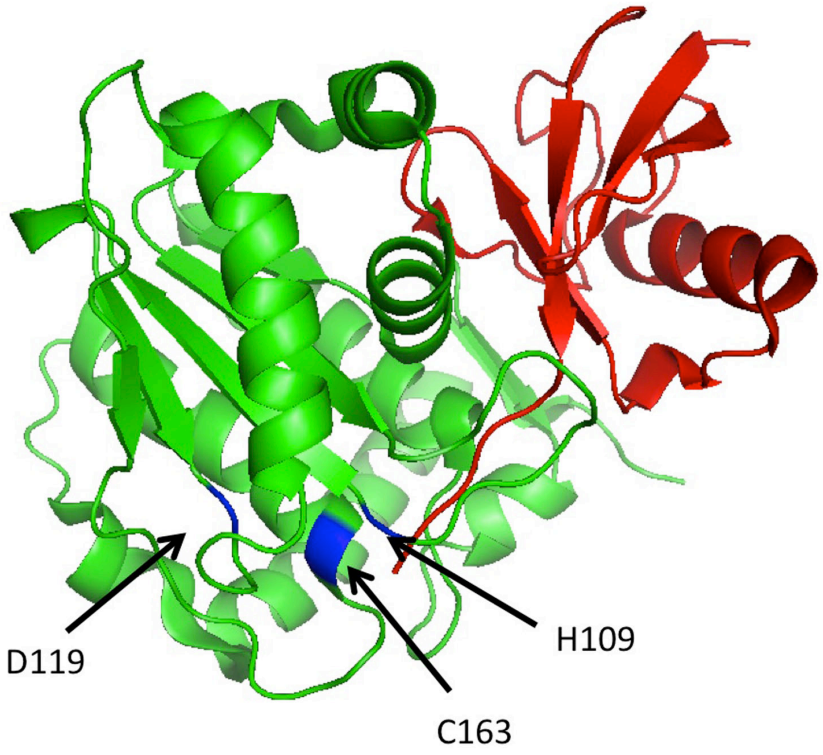


Figure 5.2: Crystal structure of the NEDP1-NEDD8 complex.

Structure was obtained from the Protein Data Bank (www.pdb.org, accession number: 2BKR) and visualized and modified in MacPymol. NEDP1 is shown in green, NEDD8 is in red, and the blue residues highlight the catalytic triad: H109, D119 and C163.

Since NEDP1 is highly specific for NEDD8 but its physiological role is not known, our lab has been interested in characterizing the enzyme. Due to the fact that NEDP1 is highly conserved between species, our lab has decided a few years ago to study the role of NEDP1 in the round worm, *C. elegans*, in collaboration with Anton Gartner's lab.

Ulp3 was identified to be the worm ortholog of NEDP1 by reciprocal Blast analysis. We tested the enzyme and found that it possesses both NEDD8 processing and deNEDDylating activity (Figure 5.3-5). Recombinant Ulp3 can process NEDD8 from a fusion protein where ubiquitin is fused at the C-terminus of NEDD8, and overexpressed Ulp3 is able to deNEDDylate L11 in human cells. The processing and deNEDDylating activity depends on the conserved catalytic cysteine residue, since mutation on Cys167 (Ulp3 Cys) inhibits both activities (Figure 5.4-5.).

The *ulp-3* deletion mutant has been obtained from the Japanese *C. elegans* knockout consortium. Given the Gartner's lab expertise in DNA damage-induced apoptosis and the existing knowledge of the response of the NEDD8 pathway to stress, we decided to investigate the effects of the *ulp-3* deletion in the DNA damage response pathway. My project addressed the role of NEDP1 in DNA-damage induced apoptosis in human cell lines.

In the following parts of this chapter I will first introduce the DNA damage-induced apoptotic pathways in worms and humans, and then give a summary on the characterization of *ulp-3*, a work that was done by Aymeric Bailly; followed by my results on NEDP1.

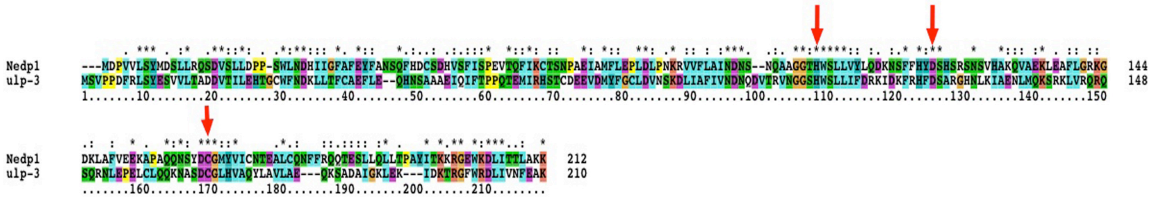


Figure 5.3: Sequence alignment of human NEDP1 and *C. elegans* ulp-3

Sequence alignment between *Homo sapiens* NEDP1 (accession number: NP_001159812.1) and *Caenorhabditis elegans* Ulp-3 (NP_001023477.1) was performed using ClustalX 2.0.12 software. The two orthologs possess 27 % sequence identity. The conserved catalytic triad residues involved in protease activity are marked with arrows. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties. A . (period) indicates conservation between groups of weakly similar properties. The residues are coloured according to their physicochemical properties (red: small + hydrophobic; blue: acidic; magenta: basic; green: hydroxyl + sulfhydryl + amine + G; grey: others).

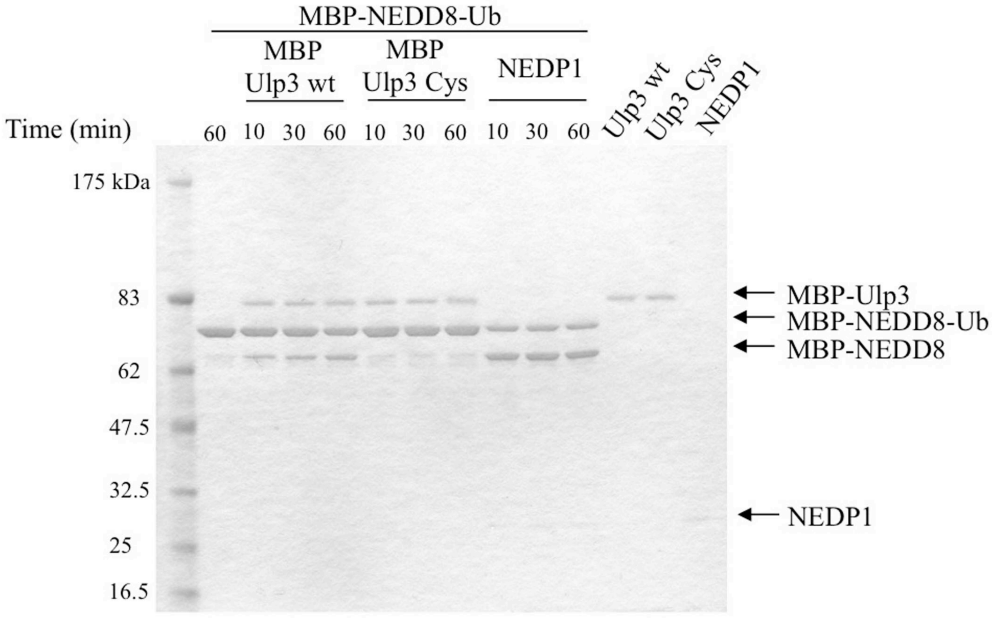


Figure 5.4: Ulp3 is a deNEDDylating enzyme, and its activity depends on the catalytic cysteine.

MBP-His-NEDD8-Ub substrate was incubated with wildtype Ulp3, Ulp3 catalytic cysteine mutant (Ulp3 Cys) and NEDP1 for the indicated time intervals *in vitro*. Proteins were analysed on SDS-PAGE gel with coomassie staining.

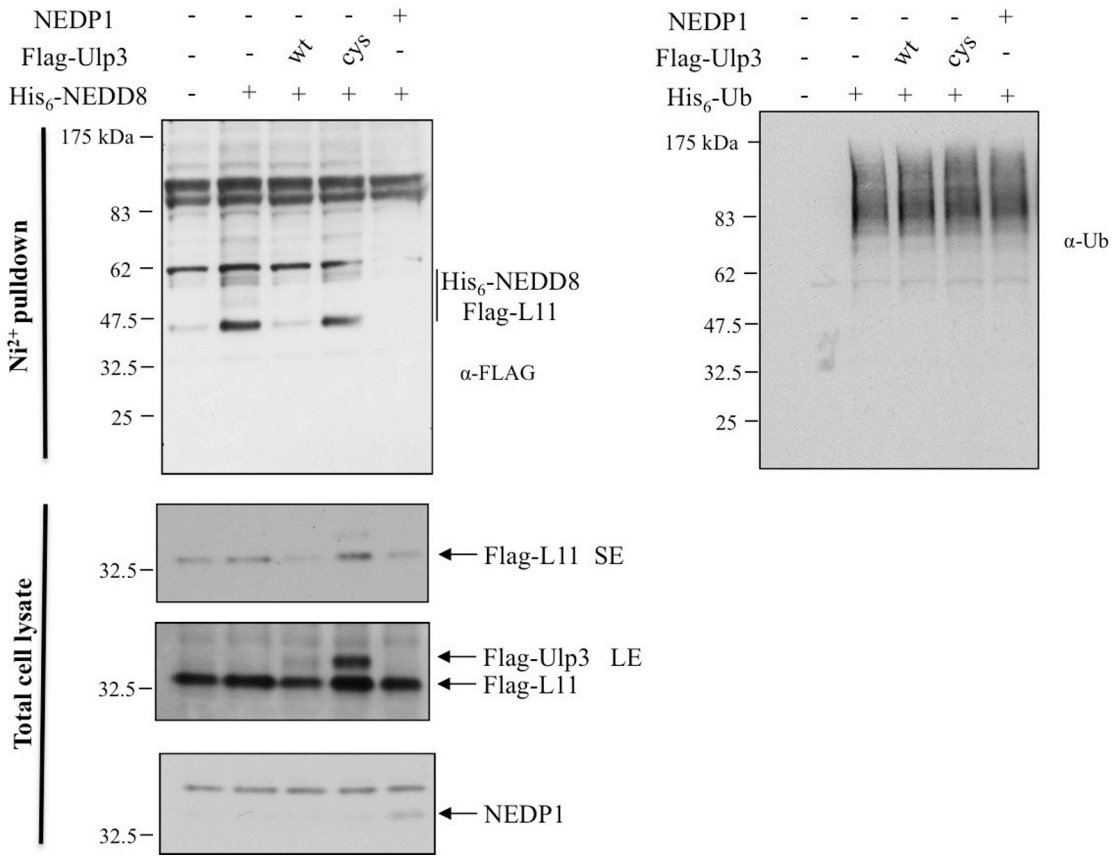


Figure 5.5: Ulp3 deNEDDylates L11 in cells, in a catalytic cysteine-dependent manner.

H1299 cells were transfected with the indicated constructs (5 µg NEDP1 or Ulp-3, 5 µg of L11 and 2 µg His-NEDD8 or His-Ub). 2 days after transfection, NEDDylated proteins were isolated with Ni^{2+} beads and analysed by western blotting. WT Ulp3 - but not the catalytic mutant- deNEDDylates L11 similarly to the human NEDP1, and does not affect ubiquitin conjugates.

5.1.2. DNA damage-induced apoptosis in worms and humans

Caenorhabditis elegans has been extensively used as a multi-cellular model organism to investigate the DNA damage response, since it is easy to grow, manipulate and it recapitulates the DNA damage response present in higher eukaryotic cells. The basic genetic regulation of apoptosis has been uncovered mainly through studies on *C. elegans*, taking advantage of the invariant development of the worm that allows tracing the fate of every single cell[305]. The induction of apoptosis in germ cells in the nematode *C. elegans* by genotoxic stress was first reported in 2000[306]; and since then it has been extensively studied.

CEP-1 (C. elegans-p53), the only p53-like protein in *C. elegans* has been shown to be required for DNA damage-induced apoptosis, and is induced by a checkpoint signaling pathway[307]. Once activated, CEP-1 induces the transcription of *egl-1* and *ced-13,* which encode for two pro-apoptotic BH3-only proteins that act upstream of the genes *ced-9, ced-4* and *ced-3[308]*. *ced-9* encodes for a Bcl-2-like cell death inhibitor*, ced-4* for an Apaf-1-like adaptor protein and *ced-3* for a protease that is related to the caspase family of proteins (Figure 5.6)[309-311].

While the core apoptotic machinery is similar between worms and humans, there are some fundamental differences as well. The pathway is more complex in mammals, with a number of BH3-only domain and Bcl-2 (B-cell lymphoma 2) proteins playing role in apoptosis along with multiple caspases. Basically, in mammals Bcl-2 inhibits apoptosis by preventing the release of cytochrome c from mitochondria that activates Apaf-1 (apoptotic protease-activating factor 1). Once cytochrome c is released, it binds to the WD40 repeats of Apaf-1 to facilitate assembly of the apoptosome in the presence of dATP/ATP. The apoptosome is a wheel-like complex that recruits procaspase-9 to form

a functional complex that is able to activate procaspase-3[312]. In worms however, cytochrome c is thought to have no role in apoptosis, and CED-4 is mainly inhibited by physical interaction with CED-9 that is disrupted upon *egl-1* activation. However, there are indications that the regulation of CED-4 may be more complex than previously thought[313].

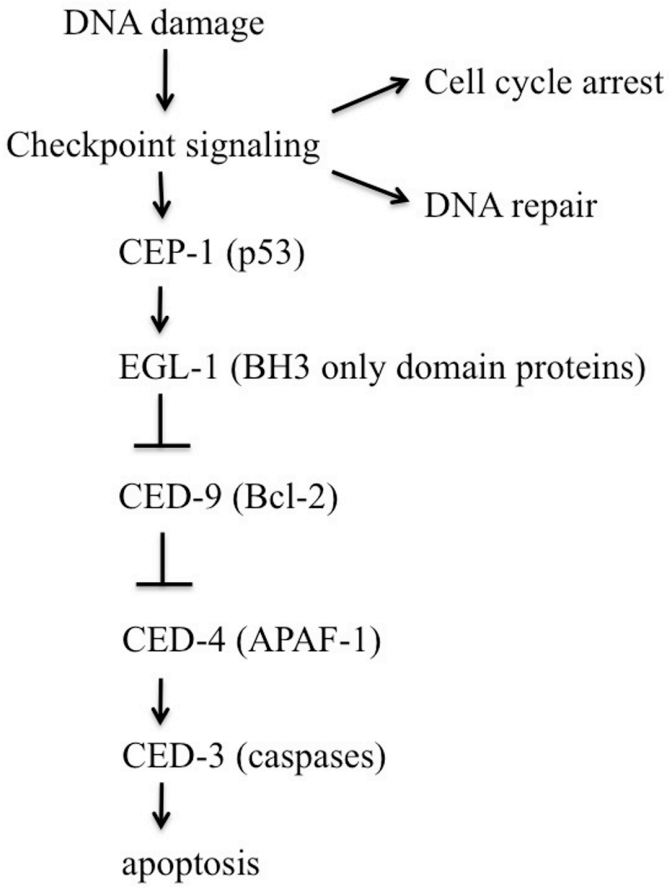


Figure 5.6: Genetic pathway in *C. elegans*, leading from DNA damage to apoptosis. The related mammalian proteins are in brackets.

Aymeric Bailly provided genetic evidence on the importance of *ulp-3* (NEDP1 homolog in *C. elegans*) in the DNA damage response. The characterisation of the mutant showed that it is viable and develops normally with no obvious defects. However, *ulp-3*

depleted worms develop less apoptotic cells in their germ line upon ionizing irradiation, compared to the wild-type animals, similarly to a *cep-1* deleted strain. Further characterisation on the *ulp-3* deleted strain has shown that the defective apoptotic response is not due to a DNA repair defect (as determined by comparing the sensitivity of the *ulp-3* strain to IR treatment to that of the *hpr-17* (homologue of pombe RAD17) mutant worms that are defective in DNA repair). CEP-1 activity was also not severely affected by the deletion of the NEDP1 ortholog. These data suggest that *ulp-3* specifically affects DNA damage-induced apoptosis in the germ line, and acts parallel or downstream of *cep-1*.

The aim of my project was to test the conservation of the role of Ulp-3 in the DNA damage-induced apoptosis in human cells and to investigate whether it acts downstream of p53.

5.2. Results

5.2.1. *nedp1* is induced upon treatment with chemotherapeutic agents

Studies from our group showed that the protein level of NEDP1 is increased upon treatment of human cells with low doses of actinomycin D (ActD)[148]. We decided to confirm this data, and investigate the response of *nedp1* gene expression to ActD and another chemotherapeutic drug, doxorubicin (Dox) as well. Dox causes DNA damage by intercalation; therefore it is relevant to our study. MCF7 cells were treated with 1 μM doxorubicin or 5 nM ActinomycinD for the indicated time periods. RNA was isolated and qPCR analysis was performed to detect gene expression of *nedp1*. We found that the expression is increased upon treatment with either agent (Figure 5.7). Recently, a paper from Watson et al., 2009 also showed the response of NEDP1 to doxorubicin and neocarzinostatin (NCS), and claimed that SENP8 plays an important role in p53 induction by inhibiting Mdm2, in response to the drug treatments[314].

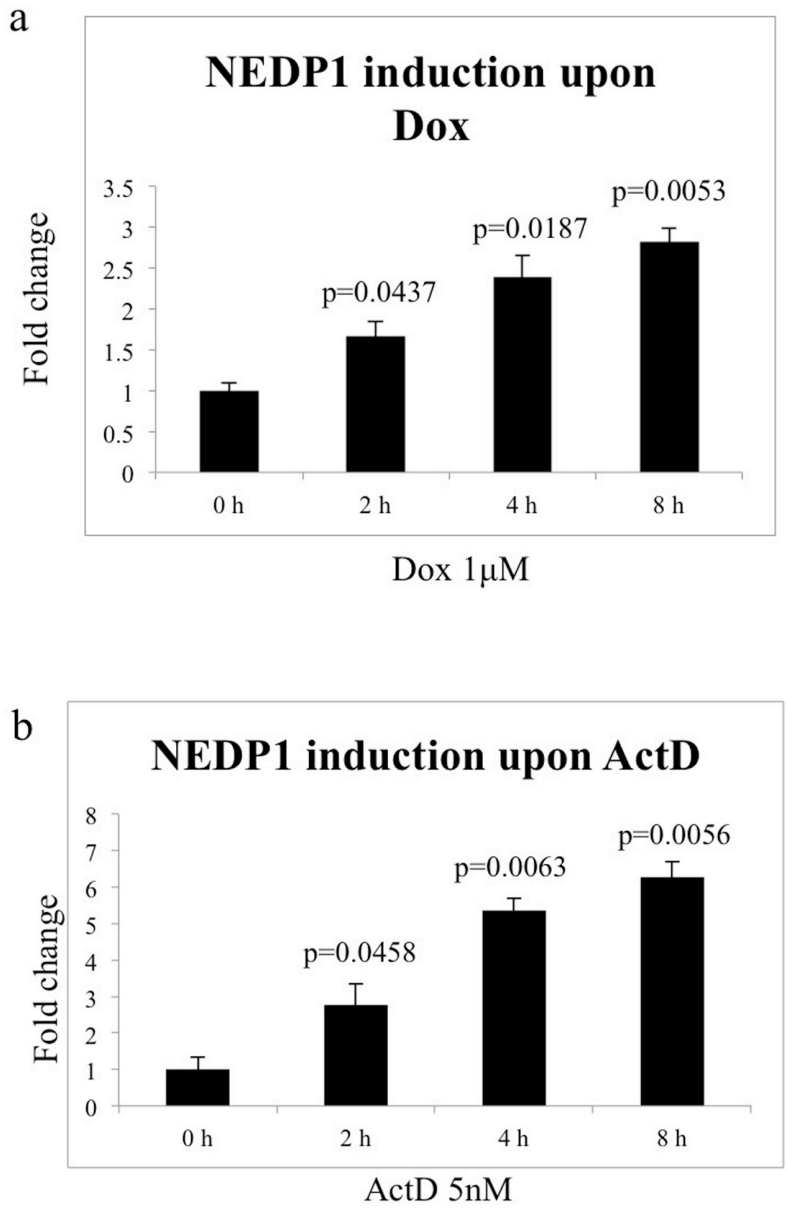


Figure 5.7*: nedp1* is induced upon doxorubicin and ActD treatment.

MCF7 cells were treated with **(a)** 1 μM doxorubicin or **(b)** 5 nM actinomycin D for the indicated time intervals. Quantitative real-time PCR for *nedp1* was carried out as described in Materials and Methods. The experiments were performed in triplicates; data are represented as mean +/-STDEV. T-test was performed for statistical significance between the 0h time point and later time points. $p<0.05$ indicates that the difference is statistically significant.

5.2.2. NEDP1 is induced upon ionizing irradiation on the mRNA and protein level

Previous data from Aymeric Bailly has shown that *ulp-3* depleted worms develop less apoptotic cells in their germ line upon ionizing irradiation, compared to the wild-type animals.

We were interested to test whether the human ortholog responds to IR. We exposed MCF7 cells to 7 or 10 Gy of IR. 4 or 24 hrs after exposure, cells were harvested. mRNA was isolated for qPCR analysis, which showed an induction in the mRNA level of the protease (Figure 5.8 a).

To determine the effect of IR on the NEDP1 protein levels, we exposed cells to 7 Gy irradiation, and lysed them in 2X SDS after 0, 4 and 24 hrs. Western blot analysis using a p53-specific antibody as a control showed that the irradiation was efficient, the protein level increased after 4 hrs and dropped at 24 hrs. The NEDP1 levels were increased but at a late time point (~24 h) (Figure 5.8.b).

Late induction in gene expression and stabilization of the gene product is a common characteristic of apoptotic genes. Our data indicate that the deNEDDylating enzyme NEDP1 responds to chemotherapeutic drugs and ionizing irradiation, and points towards a potential role in the DNA-damage response.

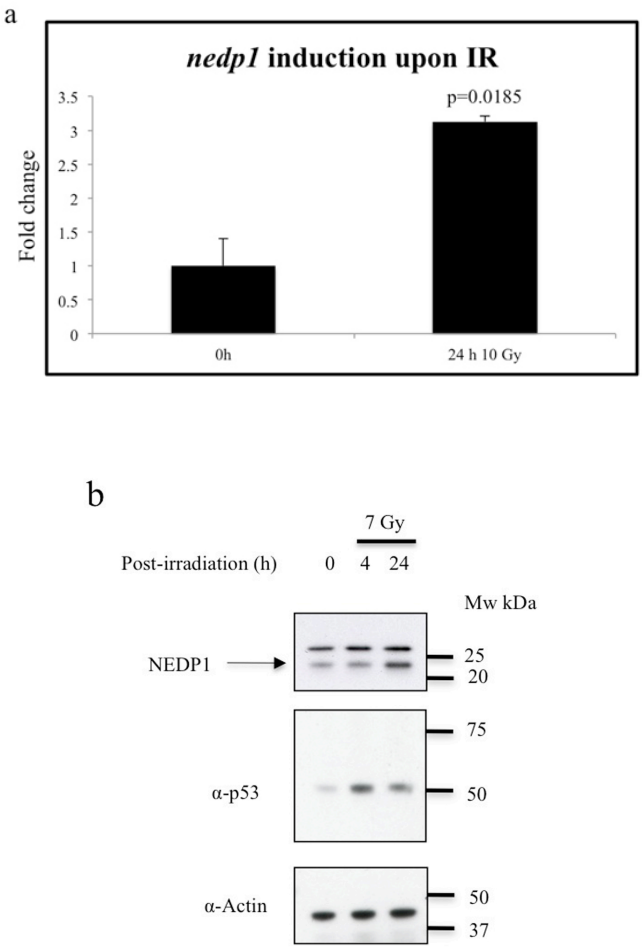


Figure 5.8: NEDP1 responds to ionizing irradiation.

(a) MCF7 cells were irradiated with 10 Gy and harvested at the indicated time. Quantitative real-time PCR for *nedp1* was carried out as described in Materials and Methods. The experiments were performed in triplicates; data are represented as mean +/-STDEV. T-test was performed for statistical significance. $p<0.05$ indicates that the difference is statistically significant. **(b)** MCF7 cells were lysed 0, 4 or 24 hrs after irradiation with 7 Gy and extracts were analysed by western blotting with the indicated antibodies.

5.2.3. Bioinformatic characterisation of the 5 NEDP1 transcript variants and prediction of the p53 binding site

Since *ulp-3* and *cep-1* deletions result in the same phenotype in response to DNA-damage, but Ulp-3 is not required for induction of CEP-1-dependent genes, we reasoned that the deNEDDylating enzyme might be itself a target for CEP-1. Therefore we decided to test whether p53 is a transcription factor for NEDP1 in mammalian cells. First we performed a bioinformatic analysis on the transcript variants of NEDP1, and predicted the p53 binding site.

The NEDP1 gene is located to chromosome 15. Alternative splicing results in 5 transcript variants. Sequence alignment between the mRNA variants showed that they are only different in their 5'UTR (Figure 5.9). All splice variants encode the same protein.

Transcription factor binding sites for the *senp8* gene were predicted with MatInspector software (www.genomatix.de). MatInspector uses a large library of matrix descriptions for transcription factor binding sites to identify matches in DNA sequences. Several transcription factors were predicted to bind to NEDP1 promoter including p53, the well-known transcription factor involved in DNA damage response. The software predicted three p53 binding sites. We therefore designed ChIP qPCR primers that amplify DNA fragments around the following predicted p53-binding site: GAGGCAAGATGTTGGCATGGGGG. This binding site is located -334 bp from the transcription start site (TSS), upstream of all the splice variants, as shown on Figure 5.10.

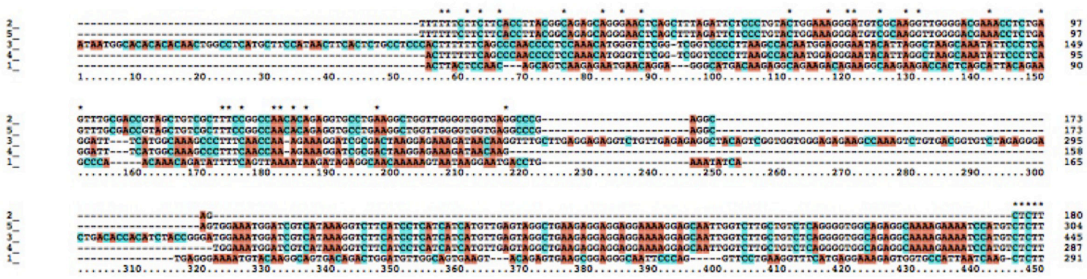


Figure 5.9: Sequence alignment between the parts of the 5'UTRs that are different.

The sequence alignment between the mRNA variants the variants (1.: accession number: NM_001166340; 2.: accession number: NM_001172111.1; 3.: accession number: NM_001172109; 4.: accession number: NM_001172110.1; 5.: accession number: NM_145204.3) was performed by ClustalX.

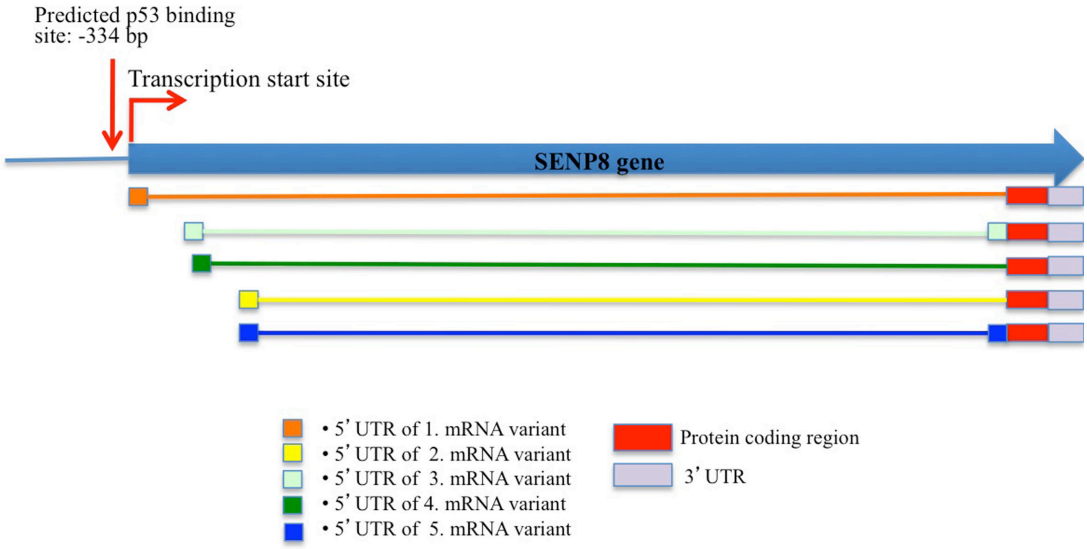


Figure 5.10: Schematic representation of the *nedp1* splice variants and the location of the predicted p53 binding site.

5.2.4. Chromatin Immunoprecipitation experiments show p53 recruitment to the *nedp1* promoter

We found that *nedp1* gene expression increases upon ActD, Dox and IR treatment. In order to test whether p53 is a regulator of the *nedp1* gene expression, we investigated the recruitment of p53 to the predicted binding site of the *nedp1* promoter by chromatin immunoprecipitation experiments. First, we treated MCF7 cells with ActD for the indicated time intervals. After harvesting, DNA was cross-linked and sheared to ~500 bp length fragments. p53 and IgG antibodies (control) were used for immunoprecipitation. ChIP primers designed to *nedp1* and *bax* promoter were used in qPCR reactions to determine whether p53 is associated with the promoters. BAX is a proapoptotic protein, a member of the Bcl2 family. *Bax* is a p53 target gene and its expression is upregulated upon DNA damage; therefore we used *bax* as a positive control in our experiment. We observed the recruitment of p53 both to the *bax* and *nedp1* promoters, indicating that p53 is indeed a transcription factor that potentially regulates *nedp1* gene expression. We next tested whether p53 is recruited to the *nedp1* promoter upon ionizing irradiation as well. This time we used HCT116 cell line, containing wild-type p53, and a p53-deficient derivative, as a negative control. Cell lines were irradiated with 7 Gy, and harvested 0, 1, 3, 5 and 8 hrs after the treatment. Chromatin immunoprecipitation was performed with p53 antibody, and the associated promoters were detected with *bax* and *nedp1* primers in a qPCR reaction. Consistent with the data obtained with ActD treatment, p53 was immunoprecipitated on the *nedp1* promoter upon IR. These data indicate that p53 can act as a transcription factor for *nedp1* and the recruitment of p53 on *nedp1* is comparable to that for *bax* (a well-described pro-apoptotic p53 target gene).

The experiments were performed with the help of Bidesh Mahata.

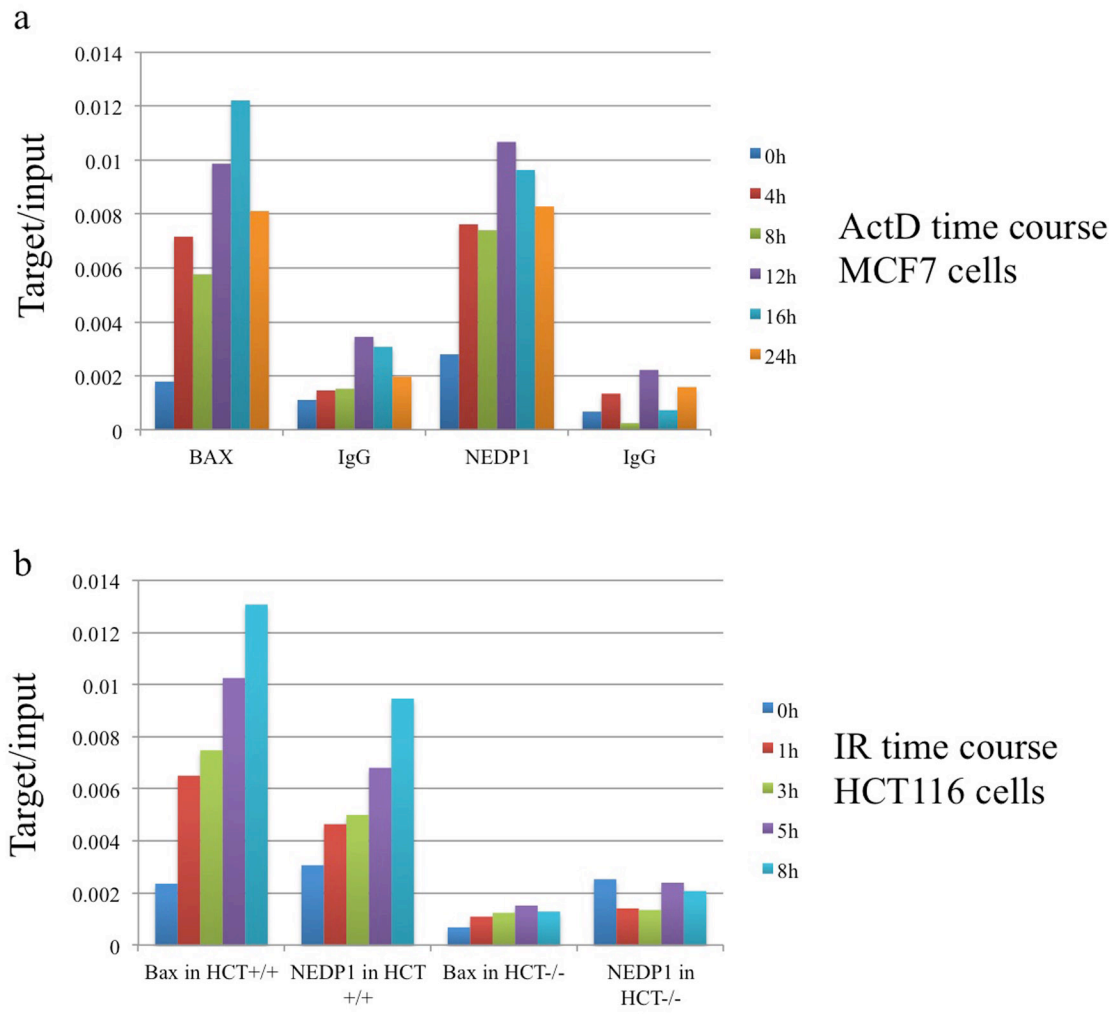


Figure 5.11: p53 occupies the *nedp1* promoter upon ActD treatment and IR

(a) MCF7 cells were treated with 5 nM ActD for the indicated time intervals. p53 and IgG antibodies (control) were used for immunoprecipitation. ChIP primers designed to *nedp1* and *bax* promoter were used in qPCR reactions to determine whether p53 is associated with the promoters. **(b)** HCT116 (+/+) or (-/-) cells were exposed to 7 Gy IR for the indicated time intervals. After immunoprecipitation with p53 antibody, the isolated DNA fragments were tested in a qPCR reaction with *nedp1* and *bax* specific primers.

5.2.5. p53 is not essential for *nedp1* gene induction upon IR

Since p53 was not the only transcription factor predicted by MatInspector, we wanted to know whether it plays an essential role in *nedp1* gene induction upon IR. We addressed this question by using the two HCT116 cell lines that are isogenic except for p53 (either p53+/+ or p53-/-). We exposed cells to 7 Gy of IR, and monitored the gene expression by qPCR 8 hrs after irradiation. We also monitored changes of a well-established p53 target gene (*p21* expression), as a positive control. The increase in *p21* gene expression upon IR was p53 dependent, since the induction could not be observed in the cell line lacking p53. However, *nedp1* was still induced in HCT116 p53-/- cell line, suggesting that p53 is not the main transcription factor for this gene. In the absence of p53, other factors can induce *nedp1* expression.

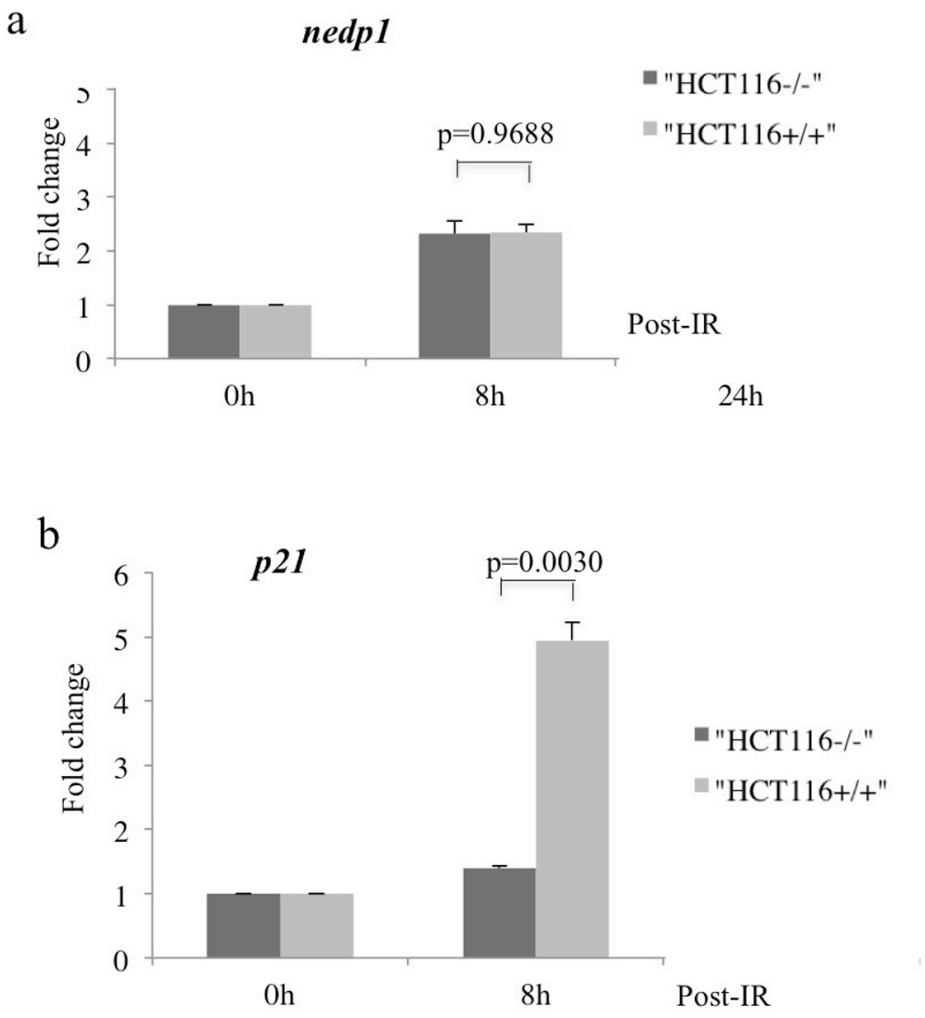


Figure 5.12: *nedp1* is induced in a p53-independent manner

HCT116 p53 negative (-/-) and positive (+/+) cells were irradiated with 7 Gy and harvested 0 and 8 hrs after irradiation. The changes in **(a)** *nedp1* and **(b)** *p21* mRNA levels were detected by quantitative real-time PCR with specific primers. T-test was performed for statistical significance between the gene inductions in the different cell lines. $p<0.05$ indicates that the difference is statistically significant.

5.2.6. NEDP1 knockdown results in impaired caspase 3/7 activation upon IR, but not upon staurosporine treatment

Ionizing irradiation triggers DNA damage-induced apoptosis. Staurosporine is a relatively non-selective protein kinase inhibitor that induces cell death in a non-genotoxic manner. Both treatments result in induction of the effector caspases. In order to test whether NEDP1 is specific for the DNA damage-induced apoptosis, we measured caspase 3/7 activity induction upon IR or staurosporine treatment in a luminescence assay, in control or NEDP1 knockdown conditions.

HCT116 cells were transfected twice within a period of 5 days either with NEDP1 or NT siRNA. 6 hrs before harvesting, cells were either left untreated, or exposed to 7 Gy ionizing irradiation or 1 μM staurosporine. Caspase activity was measured using the luminescence Caspase-Glo 3/7 Assay (Promega), according to the manufacturer's instructions. We observed a ~3- and 2,5-fold induction in caspase activity upon IR and staurosporine treatment, respectively. Knockdown of NEDP1 compromised caspase induction in response to IR while it did not have any effect on staurosporine-mediated caspase activity. This preliminary observation indicates that NEDP1 is specifically required for the DNA damage induced apoptosis; consistent with the data obtained in *C. elegans*.

A member of the lab has also performed an experiment to quantify apoptotic cells after IR and found that NEDP1 siRNA protects cells from cell death. HCT116 cells were left untreated or treated with NEDP1 siRNA and then exposed to IR. Cells were stained with Annexin V and the number of Annexin V positive cells were quantified by flow cytometry. The dye detects the externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells[315]. We have found that the number of the

apoptotic cells decreased in the absence of NEDP1 (data not shown). This result indicates that the phenotype we obtained in *C.elegans*, where *ulp-3* deletion prevents the formation of apoptotic corps in the germ line after IR treatment is also conserved in mammalian cells.

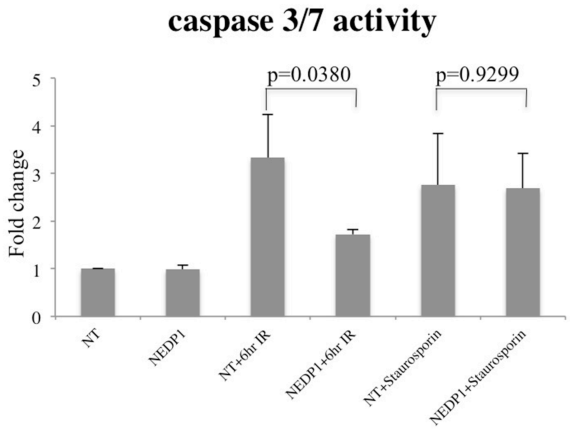


Figure 5.13: NEDP1 knockdown is required for caspase 3/7 activation upon IR, but not upon staurosporine treatment.

HCT116 cells were treated with NT or NEDP1 siRNA for 5 days. 6 hrs before harvesting, cells were either left untreated, or exposed to 7 Gy ionizing irradiation or 1 μM staurosporine. Caspase activity was measured using the luminescence Caspase-Glo 3/7 Assay (Promega). The experiment was performed in triplicates, data represent mean +/- STDEV. T-test was performed for statistical significance for the caspase activity induction with NT or NEDP1 siRNA. $p<0.05$ indicates that the difference is statistically significant. While NEDP1 siRNA significantly reduces effector caspase activation upon IR, it does not significantly affect staurosporine-induced apoptosis.

5.2.7. Testing the role of NEDP1 in caspase 8 and 9 assays induction

As mentioned in Chapter 1.10, there are two main apoptotic pathways, the extrinsic (death receptor) and the intrinsic (mitochondrial) cascade. They both converge on the same execution pathway. Caspase-8 and -9 are the initiator caspases for the extrinsic and intrinsic cascade, respectively. We tested which pathway is NEDP1 involved in by measuring caspase 8 or 9 activity after doxorubicin treatment in the presence and absence of the deNEDDylating enzyme.

A 5-days double treatment of NEDP1 or NT siRNA was performed in HCT116 cells. 6hrs before harvesting, cells were either left untreated, or treated with 1μM doxorubicin. Caspase activity was measured using Caspase-Glo 8 or 9 Assays (Promega), according to the manufacturers instructions (Figure 5.14). We have also performed the experiment with IR-treated cells and measured Caspase 8 and 9 activities 15 hrs after IR (data not shown).

NEDP1 knockdown did not significantly affect caspase induction in any cases. This result might suggest that NEDP1 acts downstream from the initiator caspases, possible directly or indirectly on the effector caspases.

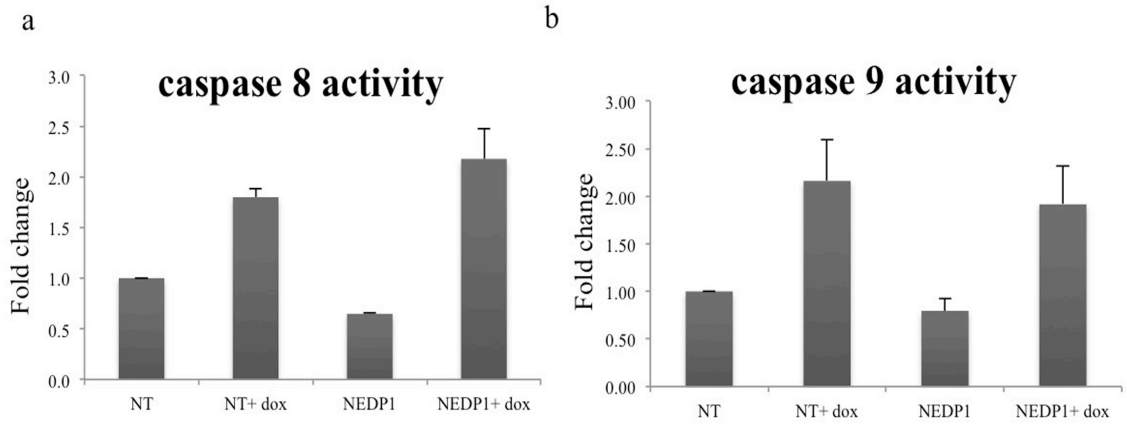


Figure 5.14: NEDP1 knockdown doesn't compromise caspase 8 or 9 induction.

A 5-days double treatment of NEDP1 or NT siRNA was performed in HCT116 cells. 6 hrs before harvesting, cells were either left untreated, or treated with 1 μM doxorubicin. Caspase activity was measured using the luminescence-based Caspase-Glo 8 or 9 Assays (Promega). The experiment was performed in triplicates, data represent mean +/- STDEV.

5.2.8. NEDP1 knockdown results in accumulation of NAE-dependent, yet to be identified NEDD8 substrates

To date, targets for NEDP1 under entirely endogenous conditions have not been identified. In order to understand the role of the enzyme, we need to find targets for its deNEDDylating activity. First we wanted to monitor protein NEDDylation upon knockdown of NEDP1. We therefore treated U2OS cells with siNEDP1 for 5 days (with the double-transfection procedure described in the Methods), lysed them with 2X SDS, and detected the NEDD8 conjugates by western blotting. Indeed, knockdown of NEDP1 resulted in the increase of distinct NEDD8 bands; mainly in the molecular size range lower than cullins. Since we have shown that not only NAE, but also Ube1 can activate

NEDD8, we were interested to test whether the substrates controlled by NEDP1 are NEDDylated through the canonical or the ubiquitin-conjugation pathway. We performed the knockdown experiment in the presence of MLN4924 or siUbe1, and found that NEDD8 modification of NEDP1 substrate proteins are promoted entirely by the canonical (NAE-mediated) NEDD8 conjugation pathway.

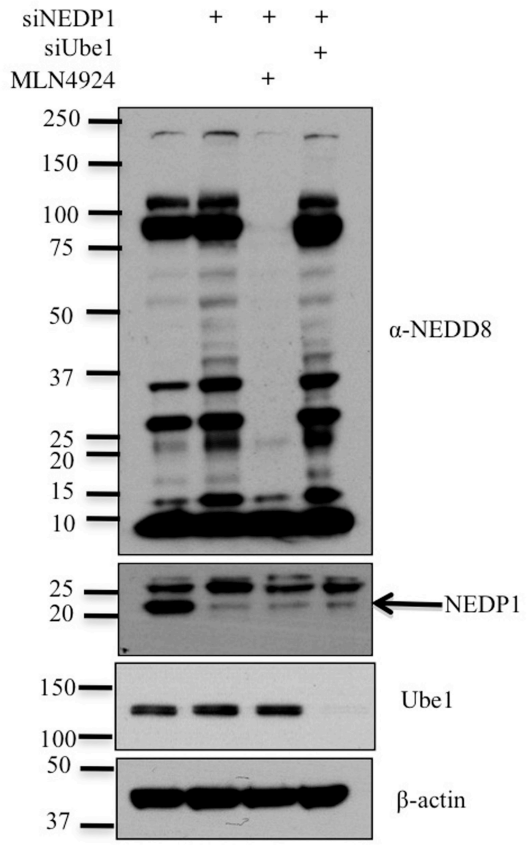


Figure 5.15: NEDP1 substrates are NEDDylated through the canonical NEDD8 pathway.

U2OS cells were transfected with the indicated siRNAs, then treated or not with 1 μM MLN4924 for 16 hrs. Cell extracts were analyzed by western blotting.

5.2.9. Doxorubicin treatment does not result in global NEDD8 response

In order to see how NEDD8 responds to DNA damage, we have also performed a western blot analysis on samples derived from U2OS cells that were treated or not with the DNA damaging agent doxorubicin. We did not detect any obvious change in the NEDDylation profile in response to the drug, suggesting that DNA damage does not result in a global NEDD8 response that can be detected by western blotting.

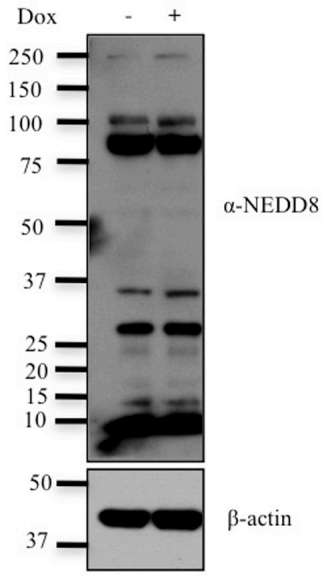


Figure 5.16: NEDD8 does not respond globally to doxorubicin treatment.

U2OS cells were treated with 1 μM doxorubicin for 16 hrs before being lysed and analyzed by western blotting.

5.2.10. Cul4A and Cul1 NEDDylation are not affected by NEDP1 knockdown

Since the substrates controlled by NEDP1 are NAE-dependent, we wanted to test whether the main NAE targets, cullins are also affected by NEDP1 siRNA. Cul1 and Cul4A have been reported to be involved in DNA damage responses. Moreover, in *C. elegans*, the SCF ligase complex has been shown to control apoptosis through regulating CEP-1[124]. Therefore we reanalysed the samples from Figure 5.15 and compared the NEDDylation status of the cullins in controlled, MN4924-or siNEDP1 treated conditions. Our data showed that NEDP1 knockdown did not affect NEDDylation of Cul4A and Cul1, while MLN4924 as expected completely inhibited them.

It would be interesting to test how NEDP1 siRNA affects CRL activity, not only their NEDDylation status. The extracts could be analysed with antibodies specific for CRL substrates, such as p21 or Cyclin E for Cul1 and DDB2 for Cul4A.

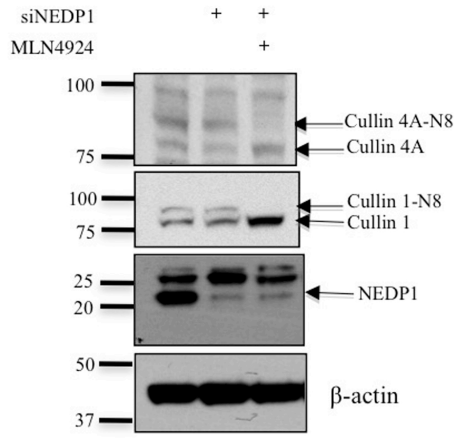


Figure 5.17: NEDP1 knockdown doesn't affect Cul4A and Cul1 NEDDylation.

U2OS cells were transfected with the indicated siRNAs, then treated or not with 1 μM MLN4924 for 16 hrs. Cell extracts were analyzed by western blotting.

5.3. Discussion and future directions

We have shown that NEDP1 is induced upon DNA damage and required for the activation of the effector caspase 3 and 7. We have also found that p53 can act as a transcription factor for *nedp1*, however its role in *nedp1* induction is not indispensible. NEDP1 most likely affects a downstream protein of the cascade, or parallel to the intrinsic apoptotic pathway.

Since the core apoptotic pathway in worms and humans is conserved, we decided to look for a target for the deNEDDylase activity of NEDP1 within the orthologs of CED-9, CED-4 and CED-3. We have tested if Bcl-2 (~CED-9), Apaf-1 (~CED-4) and caspase 2, 3 and 7 (~CED-3) are NEDDylated. We have created an HCT116 cell line stably expressing low levels of His_6NEDD8, and isolated NEDDylated proteins from untreated, IR or siNEDP1 treated cells. After Ni^{2+}-purification, we analysed the samples with Bcl-2, Apaf-1 and caspase 2,3,7 specific antibodies. However, we failed to detect the endogenous proteins from the pulldowns in the conditions used. NEDP1 might not act directly on any of these proteins, but rather affects the pathway indirectly. Since the formation of the apoptosome is crucial for the activation of effector caspases, it would be interesting to test in a gel filtration assay whether knockdown of the deNEDDylating enzyme disrupts the complex.

During my PhD, Broemer et. al has shown that DEN-1 (NEDP1 ortholog in *Drosophila*) activates the effector caspase drICE by removing NEDD8 from the protein[104]. The NEDDylation of drICE was later shown to be Ube1-dependent. Whether or not endogenous DEN-1 has an effect on the caspase-7 ortholog was not investigated in the paper.

In order to find endogenous targets for NEDP1's deNEDDylase activity, the best would be to use an unbiased approach, such as mass spectrometry-based proteomics. The method for the identification of NEDDylation sites with a R74K NEDD8 stable cell line mentioned in Chapter 4.3 could be used in a SILAC experiment. A labelled cell population could be treated with siRNA or shRNA for NEDP1, and the regulated proteins/ NEDDylation sites could be identified by mass spectrometry. Since NEDP1 knockdown results in the accumulation of lower molecular weight NEDD8 conjugates, recombinant Rpn10 might be also a good tool to isolate them under endogenous conditions. However, only partial NEDP1 knockdown can be achieved with the siRNA approach. Therefore it would be advantageous to isolate NEDDylated proteins from worms from a *ulp-3* deletion background, and compare them to wild-type animals in a SILAC experiment[316].

NEDP1 might not affect directly the core apoptotic pathway. In mammalian cells, there are many additional factors that play role in regulating the signalling cascades that lead to apoptosis. The transcription factor E2F-1 has been shown to be induced in response to DNA damage[317], and is important for the activation of the *p73* proapoptotic target gene[318]. Two recent studies demonstrated that E2F-1 is posttranslationally modified by NEDD8[150, 151]. Aoki et al has also shown that knockdown of NEDP1 attenuated *p73* expression induced by E2F-1 and abolished apoptosis. This result fits to our observations that the deNEDDylating enzyme is required for DNA damage-induced apoptosis and might explain the phenotype in mammalian cells, but not in *C. elegans*. The worm ortholog of E2F-1, EFL-1 is not required for DNA damage-induced germ cell apoptosis[319], therefore the apoptotic defect cannot be explained by the suppositional EFL-1 inhibition by *ulp-3* deletion.

It is also a possibility that the NEDD8 accumulation seen on the western blot after NEDP1 knockdown does not represent NEDDylated substrates, but indicates the

appearance of unanchored chains in the cells. As discussed in the Introduction and Chapter 4.3, these free chains can play important biological roles such as providing platforms for protein interactions. NEDP1 might control the formation of such polymers by eliminating them. It would be interesting to test whether NEDP1 is able to disassemble NEDD8 chains from the proximal end, like it was shown in the case of Usp5 and free ubiquitin chains.

Chapter 6: Final discussion

The work described in this thesis expanded the scope of NEDD8 conjugation. Historically, NEDD8 has been thought to influence the ubiquitin system via the regulation of CRLs through a covalent modification that is mediated by a specific set of NEDD8 activating and conjugating enzymes. This is thought to be the exact function of NEDD8 that is targeted therapeutically by MLN4924. However, as described in this thesis, we have discovered a more profound interplay between NEDD8 and ubiquitin, where the ubiquitin E1 activating enzyme Ube1 activates NEDD8 under stress conditions such as proteasome inhibition, heat shock and oxidative stress. NEDD8 engagement into the ubiquitin conjugation system results in mixed chain formation that is recognized by ubiquitin receptors. This discovery has expanded the function of protein regulation by NEDD8 beyond CRLs, and also raised many questions. What is the biological function of Ube1-mediated NEDDylation? What are the targets? How are the NEDD8/ubiquitin mixed chains recognized and processed in cells?

Determining the biological consequences of Ube1-mediated NEDDylation would not only be interesting, but might be therapeutically relevant. The proteasome inhibitor velcade has been used in clinics for the treatment of multiple myeloma and mantle cell lymphoma. Moreover, it is likely to be used for other applications in the future, for instance to deplete normal antibody-producing plasma cells in transplant patients to prevent from antibody-mediated rejection. Therefore, it would be important to determine the consequence of elevated NEDDylation in patient treatment, or how it might affect combinatorial therapy with other drugs. For instance, we showed that the combination of MG132 and MLN4924 results in an even more enhanced Ube1-mediated NEDDylation. By inhibiting NEDDylation of cullins, the major NEDD8 targets, MLN4924 “frees up” a large proportion of conjugable NEDD8 that is used by Ube1 if a trigger occurs. Paradoxically, inhibition of the canonical NEDD8 conjugation cascade results in increased NEDDylation by the alternative pathway under certain

circumstances. MLN4924 is now in clinical phase II trial for the treatment of acute myeloid leukemia, and reports on potential applications for the treatment of other types of cancer have been appearing recently. Whether the increase in Ube1-mediated NEDDylation on non-cullin substrates by MG132 and MLN4924 would have any effect (positive or negative) on cancer treatment has to be determined.

In addition to cancer therapy, Ube1-mediated NEDDylation can be linked to disease conditions that involve decreased proteasome activity. The ubiquitin proteasome system plays a key role as a quality control pathway that removes misfolded, or damaged proteins. The cellular insults studied in this thesis, heat shock and oxidative stress, have a well-defined effect on generating misfolded or oxidised damaged proteins and their removal by the UPS is critical for the cells to recover from these insults[320]. What is also evident is that the age-related decrease in proteasome activity observed in many tissues is associated with the accumulation of misfolded and/or oxidised damaged proteins. These observations are linked to the development of pathology including cardiac dysfunction, cataract formation and neurodegenerative diseases such as Alzheimer's and Parkinson's[320]. NEDD8 and ubiquitin also accumulate under pathological conditions in areas of protein aggregation such as Lewy bodies in Parkinson's disease, Rosenthal fibres in astrocytomas and Mallory bodies in alcoholic liver disease [321, 322]. Our studies provide a possible mechanistic link of how perturbations in proteasomal activity could result in the accumulation of NEDD8 conjugates observed in many pathological conditions.

We have characterized NEDD8 in another therapeutically relevant stress response, the DNA damage-induced apoptosis. Our group in collaboration with Anton Gartner's lab has found that the *C. elegans* ortholog of the deNEDDylating enzyme NEDP1, ulp-3 is required for DNA damage-induced apoptosis in the germ line. We have shown that the role of NEDP1 is conserved between worms and humans: NEDP1 is induced in

response to IR in the mRNA and protein levels, and is required for the induction of effector caspase activity. Annexin V staining has also confirmed that NEDP1 siRNA protects cells from cell death after IR, consistent with the phenotype observed in *C. elegans*. We found p53 as a potential transcription factor controlling *nedp1* gene expression, however, it is not required for *nedp1* induction upon IR. We determined that NEDP1 is specifically required for DNA damage-induced apoptosis, as NEDP1 siRNA had no effect on caspase 3/7 activity induced by staurosporine, a non-genotoxic agent that induces apoptosis.

Even though NEDD8 does not respond globally to DNA damaging agents such as doxorubicin treatment or IR (as observed from western blots), knockdown of NEDP1 results in the accumulation of NEDD8 conjugates that are dependent on NAE. As discussed in chapter 5.3, these conjugates have to be further investigated. First of all, it would be interesting to know whether they are unanchored NEDD8 chains, or substrate proteins. All of the bands detected on the western blot are lower molecular weight than cullins, suggesting that NEDP1 controls NEDDylation of non-cullin substrates. The lower molecular weight bands have been suggested to be degraded cullin complexes, a hypothesis that needs to be tested. However, since NEDP1 possesses little deNEDDylating activity towards cullins *in vivo* in model organisms, its targets most likely are other NAE-dependent NEDD8 substrates. For the identification of NEDP1 targets, an unbiased, site-specific proteomics approach would be the most suitable, preferably performed in a model organism on a NEDP1 knockout genetic background. The data from the *C.elegans* studies show that NEDP1 is not an essential gene, which allows proteomic studies on the NEDP1 knockout background.

NEDDylation has become a rapidly expanding field since the development of the NAE-inhibitor MLN4924. The utilization of MLN4924 makes it possible to study the classical NEDD8 pathway in diverse tumors and animal model systems in context with cancer therapy. The number of studies appearing since MLN4924 is available for research use is increasing, and they indicate a very complex regulation of protein homeostasis by a single modification, NEDD8. Until recently, NEDD8 has been thought to conjugate exclusively to cullins and a handful of other substrates. So far, there have been only a few substrates identified under endogenous conditions to be NEDDylated by the canonical NEDD8 pathway: cullins and VHL. Additionally, NEDD8 modification on p53 and HIF1α has been shown to be mediated by both the NAE and Ube1. However, we observed that proteins distinct from cullins are also regulated by MLN4924, such as the ones that appear on the western blot after NEDP1 knockdown.

In addition, we have discovered that there are NEDD8 substrates that depend on the ubiquitin conjugation cascade, further expanding the NEDD8 proteome. Since for a long time the only well-established role for NEDD8 was the regulation of CRLs, and Hjerpe et al. has shown that Ube1 activates NEDD8 when the ubl is overexpressed[260], the Ube1-mediated NEDD8 conjugation has been so far referred to as “erroneous conjugation of NEDD8”, “misactivation” or “faulty neddylation”[323, 324]. These labels indicate that the scientific community regards the mechanism as an artifact; regardless we showed that the NEDD8 activation by Ube1 occurs endogenously, under physiological stress conditions. In my opinion this novel type of NEDDylation should not be labeled as an error, it should rather be investigated open-mindedly. I would rather term the phenomenon as alternative, non-canonical or Ube1-mediated NEDDylation. I do agree to avoid overexpression whenever it is possible, and interpret the data obtained from overexpression experiments with caution. When a new

NEDD8 target is found, the manner of NEDDylation should be determined and classified as NAE-/or Ube1 target. However, Ube1 targets should not be regarded as experimental artifacts. Even though we don't have a proof that this type of NEDDylation is biologically significant, we cannot exclude it either.

The number of groups studying NEDD8-ubiquitin chains and Ube1-dependent NEDDylation is rapidly increasing. Research in the last few years has opened a door to a greater understanding of protein regulation by NEDD8, and left the research community with numerous exciting mechanistic and functional questions to be answered. Taken together, our studies indicate that the function of NEDD8 extends beyond the regulation of Cullin Ring Ligase complexes. The new challenges in the field are: 1, to determine non-cullin substrates for the canonical NEDD8 pathway and NEDP1 2, determine substrates for NEDDylation through the ubiquitin enzymes and identify the enzymes that control the non-canonical pathway 3, investigate the biological relevance of the non-canonical NEDD8 conjugation (for example how the degradation machinery recognizes and most importantly deals with the mixed chains).

When the yeast NEDD8, Rub1 was found to be conjugated to cdc53 in *Saccharomyces cerevisiae,* the discovery was initially greeted cautiously. The reason for it was that Rub1 deletion mutants in budding yeast were 'distressingly healthy'. Only later on, when studied in different genetic organisms, the functional importance of the NEDD8 pathway became evident[325]. Maybe the same 'enlightenment' will come in connection with the Ube1-mediated NEDDylation, once we find the right tool to study it?

References

1. Goldstein G, Scheid M, Hammerling U, Schlesinger DH, Niall HD & Boyse EA (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci U S A* **72**, 11-15.

2. Ciehanover A, Hod Y & Hershko A (1978) A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem Biophys Res Commun* **81**, 1100-1105.

3. Hershko A, Ciechanover A, Heller H, Haas AL & Rose IA (1980) Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc Natl Acad Sci U S A* **77**, 1783-1786.

4. Ciechanover A, Heller H, Elias S, Haas AL & Hershko A (1980) ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc Natl Acad Sci U S A* **77**, 1365-1368.

5. Ciechanover A, Elias S, Heller H & Hershko A (1982) "Covalent affinity" purification of ubiquitin-activating enzyme. *J Biol Chem* **257**, 2537-2542.

6. Hershko A, Heller H, Elias S & Ciechanover A (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* **258**, 8206-8214.

7. Baumeister W, Walz J, Zuhl F & Seemuller E (1998) The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-380.

8. Ciechanover A, Finley D & Varshavsky A (1984) Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. *Cell* **37**, 57-66.

9. Jentsch S, McGrath JP & Varshavsky A (1987) The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature* **329**, 131-134, doi: 10.1038/329131a0.

10. Hochstrasser M & Varshavsky A (1990) In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. *Cell* **61**, 697-708.

11. Finley D, Ozkaynak E & Varshavsky A (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell* **48**, 1035-1046, doi: 0092-8674(87)90711-2 [pii].

12. Goldknopf IL & Busch H (1977) Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc Natl Acad Sci U S A* **74**, 864-868.

13. Xu M, Skaug B, Zeng W & Chen ZJ (2009) A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. *Mol Cell* **36**, 302-314, doi: 10.1016/j.molcel.2009.10.002.

14. Schulman BA & Harper JW (2009) Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Biol* **10**, 319-331, doi: nrm2673 [pii]

10.1038/nrm2673.

15. Lake MW, Wuebbens MM, Rajagopalan KV & Schindelin H (2001) Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. *Nature* **414**, 325-329, doi: 10.1038/35104586

35104586 [pii].

16. Duda DM, Walden H, Sfondouris J & Schulman BA (2005) Structural analysis of Escherichia coli ThiF. *J Mol Biol* **349**, 774-786, doi: S0022-2836(05)00410-9 [pii]

10.1016/j.jmb.2005.04.011.

17. Lehmann C, Begley TP & Ealick SE (2006) Structure of the Escherichia coli ThiS-ThiF complex, a key component of the sulfur transfer system in thiamin biosynthesis. *Biochemistry* **45**, 11-19, doi: 10.1021/bi051502y.

18. Jin J, Li X, Gygi SP & Harper JW (2007) Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. *Nature* **447**, 1135-1138, doi: nature05902 [pii]

10.1038/nature05902.

19. Pickart CM (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**, 503-533, doi: 10.1146/annurev.biochem.70.1.503.

20. Eddins MJ, Carlile CM, Gomez KM, Pickart CM & Wolberger C (2006) Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. *Nat Struct Mol Biol* **13**, 915-920, doi: nsmb1148 [pii]

10.1038/nsmb1148.

21. Hershko A, Heller H, Elias S & Ciechanover A (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* **258**, 8206-8214.

22. Scheffner M, Nuber U & Huibregtse JM (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**, 81-83, doi: 10.1038/373081a0.

23. Scheffner M, Werness BA, Huibregtse JM, Levine AJ & Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129-1136.

24. Deshaies RJ & Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78**, 399-434, doi: 10.1146/annurev.biochem.78.101807.093809.

25. Pruneda JN, Littlefield PJ, Soss SE, Nordquist KA, Chazin WJ, Brzovic PS & Klevit RE Structure of an E3:E2 approximately Ub Complex Reveals an Allosteric Mechanism Shared among RING/U-box Ligases. *Mol Cell*, doi: S1097-2765(12)00591-6 [pii]

10.1016/j.molcel.2012.07.001.

26. Plechanovova A, Jaffray EG, Tatham MH, Naismith JH & Hay RT Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature*, doi: nature11376 [pii]

10.1038/nature11376.

27. Hoppe T (2005) Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. *Trends Biochem Sci* **30**, 183-187, doi: 10.1016/j.tibs.2005.02.004.

28. Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU & Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635-644.

29. Komander D, Clague MJ & Urbe S (2009) Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol* **10**, 550-563, doi: nrm2731 [pii]

10.1038/nrm2731.

30. Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, Ookata K, Nureki O, Iwai K, Komada M & Fukai S (2008) Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* **455**, 358-362, doi: 10.1038/nature07254.

31. Hu M, Li P, Li M, Li W, Yao T, Wu JW, Gu W, Cohen RE & Shi Y (2002) Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde. *Cell* **111**, 1041-1054.

32. Cao J & Yan Q (2012) Histone ubiquitination and deubiquitination in transcription, DNA damage response, and cancer. *Front Oncol* **2**, 26, doi: 10.3389/fonc.2012.00026.

33. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B & Yarden Y (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**, 3663-3674.

34. Strous GJ, van Kerkhof P, Govers R, Ciechanover A & Schwartz AL (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *Embo J* **15**, 3806-3812.

35. Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M & D'Andrea AD (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* **7**, 249-262.

36. Strack B, Calistri A, Accola MA, Palu G & Gottlinger HG (2000) A role for ubiquitin ligase recruitment in retrovirus release. *Proc Natl Acad Sci U S A* **97**, 13063-13068, doi: 10.1073/pnas.97.24.13063.

37. Ikeda F & Dikic I (2008) Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep* **9**, 536-542, doi: embor200893 [pii]

10.1038/embor.2008.93.

38. Petroski MD & Deshaies RJ (2005) Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* **123**, 1107-1120, doi: 10.1016/j.cell.2005.09.033.

39. Williamson A, Wickliffe KE, Mellone BG, Song L, Karpen GH & Rape M (2009) Identification of a physiological E2 module for the human anaphase-promoting complex. *Proc Natl Acad Sci U S A* **106**, 18213-18218, doi: 10.1073/pnas.0907887106.

40. Christensen DE, Brzovic PS & Klevit RE (2007) E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages. *Nat Struct Mol Biol* **14**, 941-948, doi: 10.1038/nsmb1295.

41. Kim HC & Huibregtse JM (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity. *Mol Cell Biol* **29**, 3307-3318, doi: 10.1128/MCB.00240-09.

42. Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK & Varshavsky A (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576-1583.

43. Kaiser SE, Riley BE, Shaler TA, Trevino RS, Becker CH, Schulman H & Kopito RR (2011) Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. *Nat Methods* **8**, 691-696, doi: 10.1038/nmeth.1649.

44. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, et al. Systematic and Quantitative Assessment of the Ubiquitin-Modified Proteome. *Mol Cell*, doi: S1097-2765(11)00675-7 [pii]

10.1016/j.molcel.2011.08.025.

45. Mukhopadhyay D & Riezman H (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **315**, 201-205, doi: 10.1126/science.1127085.

46. Chen ZJ & Sun LJ (2009) Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* **33**, 275-286, doi: 10.1016/j.molcel.2009.01.014.

47. Wickliffe KE, Williamson A, Meyer HJ, Kelly A & Rape M (2011) K11-linked ubiquitin chains as novel regulators of cell division. *Trends Cell Biol* **21**, 656-663, doi: 10.1016/j.tcb.2011.08.008.

48. Matsumoto ML, Wickliffe KE, Dong KC, Yu C, Bosanac I, Bustos D, Phu L, Kirkpatrick DS, Hymowitz SG, Rape M, et al. (2010) K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol Cell* **39**, 477-484, doi: 10.1016/j.molcel.2010.07.001.

49. Dynek JN, Goncharov T, Dueber EC, Fedorova AV, Izrael-Tomasevic A, Phu L, Helgason E, Fairbrother WJ, Deshayes K, Kirkpatrick DS, et al. (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. *Embo J* **29**, 4198-4209, doi: 10.1038/emboj.2010.300.

50. Sobhian B, Shao G, Lilli DR, Culhane AC, Moreau LA, Xia B, Livingston DM & Greenberg RA (2007) RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science* **316**, 1198-1202, doi: 10.1126/science.1139516.

51. Wagner SA, Beli P, Weinert BT, Nielsen ML, Cox J, Mann M & Choudhary C (2011) A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol Cell Proteomics* **10**, M111 013284, doi: 10.1074/mcp.M111.013284.

52. Hwang CS, Shemorry A, Auerbach D & Varshavsky A (2010) The N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases. *Nat Cell Biol* **12**, 1177-1185, doi: 10.1038/ncb2121.

53. Hatakeyama S, Yada M, Matsumoto M, Ishida N & Nakayama KI (2001) U box proteins as a new family of ubiquitin-protein ligases. *J Biol Chem* **276**, 33111-33120, doi: 10.1074/jbc.M102755200.

54. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ & Springer W (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* **12**, 119-131, doi: 10.1038/ncb2012.

55. Al-Hakim AK, Zagorska A, Chapman L, Deak M, Peggie M & Alessi DR (2008) Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. *Biochem J* **411**, 249-260, doi: 10.1042/BJ20080067.

56. Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K & Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *Embo J* **25**, 4877-4887, doi: 10.1038/sj.emboj.7601360.

57. Rahighi S, Ikeda F, Kawasaki M, Akutsu M, Suzuki N, Kato R, Kensche T, Uejima T, Bloor S, Komander D, et al. (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* **136**, 1098-1109, doi: 10.1016/j.cell.2009.03.007.

58. Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW, et al. (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* **471**, 591-596, doi: 10.1038/nature09816.

59. Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJ, Goswami P, Nagy V, Terzic J, et al. (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. *Nature* **471**, 637-641, doi: 10.1038/nature09814.

60. Kulathu Y & Komander D Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat Rev Mol Cell Biol* **13**, 508-523, doi: nrm3394 [pii]

10.1038/nrm3394.

61. Dikic I, Wakatsuki S & Walters KJ (2009) Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol* **10**, 659-671, doi: 10.1038/nrm2767.

62. Tenno T, Fujiwara K, Tochio H, Iwai K, Morita EH, Hayashi H, Murata S, Hiroaki H, Sato M, Tanaka K, et al. (2004) Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. *Genes Cells* **9**, 865-875, doi: 10.1111/j.1365-2443.2004.00780.x.

63. Lange OF, Lakomek NA, Fares C, Schroder GF, Walter KF, Becker S, Meiler J, Grubmuller H, Griesinger C & de Groot BL (2008) Recognition dynamics up to microseconds revealed from an RDC-derived ubiquitin ensemble in solution. *Science* **320**, 1471-1475, doi: 10.1126/science.1157092.

64. Coux O, Tanaka K & Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* **65**, 801-847, doi: 10.1146/annurev.bi.65.070196.004101.

65. Fisher RD, Wang B, Alam SL, Higginson DS, Robinson H, Sundquist WI & Hill CP (2003) Structure and ubiquitin binding of the ubiquitin-interacting motif. *J Biol Chem* **278**, 28976-28984, doi: 10.1074/jbc.M302596200.

66. Hofmann K & Bucher P (1996) The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem Sci* **21**, 172-173.

67. Elsasser S & Finley D (2005) Delivery of ubiquitinated substrates to protein-unfolding machines. *Nat Cell Biol* **7**, 742-749, doi: 10.1038/ncb0805-742.

68. Wilkinson CR, Seeger M, Hartmann-Petersen R, Stone M, Wallace M, Semple C & Gordon C (2001) Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat Cell Biol* **3**, 939-943, doi: 10.1038/ncb1001-939.

69. Young P, Deveraux Q, Beal RE, Pickart CM & Rechsteiner M (1998) Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a. *J Biol Chem* **273**, 5461-5467.

70. Hofmann K (2009) Ubiquitin-binding domains and their role in the DNA damage response. *DNA Repair (Amst)* **8**, 544-556, doi: 10.1016/j.dnarep.2009.01.003.

71. Bienko M, Green CM, Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, et al. (2005) Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science* **310**, 1821-1824, doi: 10.1126/science.1120615.

72. Husnjak K, Elsasser S, Zhang N, Chen X, Randles L, Shi Y, Hofmann K, Walters KJ, Finley D & Dikic I (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* **453**, 481-488, doi: 10.1038/nature06926.

73. Kamadurai HB, Souphron J, Scott DC, Duda DM, Miller DJ, Stringer D, Piper RC & Schulman BA (2009) Insights into ubiquitin transfer cascades from a structure of a UbcH5B approximately ubiquitin-HECT(NEDD4L) complex. *Mol Cell* **36**, 1095-1102, doi: 10.1016/j.molcel.2009.11.010.

74. Husnjak K & Dikic I Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu Rev Biochem* **81**, 291-322, doi: 10.1146/annurev-biochem-051810-094654.

75. Isasa M, Katz EJ, Kim W, Yugo V, Gonzalez S, Kirkpatrick DS, Thomson TM, Finley D, Gygi SP & Crosas B (2010) Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. *Mol Cell* **38**, 733-745, doi: 10.1016/j.molcel.2010.05.001.

76. Matsumoto G, Wada K, Okuno M, Kurosawa M & Nukina N (2011) Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Mol Cell* **44**, 279-289, doi: 10.1016/j.molcel.2011.07.039.

77. Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* **78**, 477-513, doi: 10.1146/annurev.biochem.78.081507.101607.

78. Borissenko L & Groll M (2007) 20S proteasome and its inhibitors: crystallographic knowledge for drug development. *Chem Rev* **107**, 687-717, doi: 10.1021/cr0502504.

79. Navon A & Goldberg AL (2001) Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Mol Cell* **8**, 1339-1349.

80. Kang Y, Chen X, Lary JW, Cole JL & Walters KJ (2007) Defining how ubiquitin receptors hHR23a and S5a bind polyubiquitin. *J Mol Biol* **369**, 168-176, doi: 10.1016/j.jmb.2007.03.008.

81. Goldberg AL, Cascio P, Saric T & Rock KL (2002) The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Mol Immunol* **39**, 147-164.

82. Yao T & Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419**, 403-407, doi: 10.1038/nature01071.

83. Lam YA, Xu W, DeMartino GN & Cohen RE (1997) Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature* **385**, 737-740, doi: 10.1038/385737a0.

84. Leggett DS, Hanna J, Borodovsky A, Crosas B, Schmidt M, Baker RT, Walz T, Ploegh H & Finley D (2002) Multiple associated proteins regulate proteasome structure and function. *Mol Cell* **10**, 495-507.

85. Hanna J, Meides A, Zhang DP & Finley D (2007) A ubiquitin stress response induces altered proteasome composition. *Cell* **129**, 747-759, doi: 10.1016/j.cell.2007.03.042.

86. Kumar S, Tomooka Y & Noda M (1992) Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem Biophys Res Commun* **185**, 1155-1161.

87. Rabut G & Peter M (2008) Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep* **9**, 969-976, doi: embor2008183 [pii]

10.1038/embor.2008.183.

88. Gan-Erdene T, Nagamalleswari K, Yin L, Wu K, Pan ZQ & Wilkinson KD (2003) Identification and characterization of DEN1, a deneddylase of the ULP family. *J Biol Chem* **278**, 28892-28900, doi: 10.1074/jbc.M302890200

M302890200 [pii].

89. Mendoza HM, Shen LN, Botting C, Lewis A, Chen J, Ink B & Hay RT (2003) NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J Biol Chem* **278**, 25637-25643, doi: 10.1074/jbc.M212948200

M212948200 [pii].

90. Shen LN, Liu H, Dong C, Xirodimas D, Naismith JH & Hay RT (2005) Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1. *Embo J* **24**, 1341-1351, doi: 7600628 [pii]

10.1038/sj.emboj.7600628.

91. Wada H, Kito K, Caskey LS, Yeh ET & Kamitani T (1998) Cleavage of the C-terminus of NEDD8 by UCH-L3. *Biochem Biophys Res Commun* **251**, 688-692, doi: S0006-291X(98)99532-8 [pii]

10.1006/bbrc.1998.9532.

92. Linghu B, Callis J & Goebl MG (2002) Rub1p processing by Yuh1p is required for wild-type levels of Rub1p conjugation to Cdc53p. *Eukaryot Cell* **1**, 491-494.

93. Wang M, Medeiros BC, Erba HP, DeAngelo DJ, Giles FJ & Swords RT Targeting protein neddylation: a novel therapeutic strategy for the treatment of cancer. *Expert Opin Ther Targets* **15**, 253-264, doi: 10.1517/14728222.2011.550877.

94. Watson IR, Irwin MS & Ohh M NEDD8 pathways in cancer, Sine Quibus Non. *Cancer Cell* **19**, 168-176, doi: S1535-6108(11)00003-1 [pii]

10.1016/j.ccr.2011.01.002.

95. Liakopoulos D, Doenges G, Matuschewski K & Jentsch S (1998) A novel protein modification pathway related to the ubiquitin system. *Embo J* **17**, 2208-2214, doi: 10.1093/emboj/17.8.2208.

96. Osaka F, Kawasaki H, Aida N, Saeki M, Chiba T, Kawashima S, Tanaka K & Kato S (1998) A new NEDD8-ligating system for cullin-4A. *Genes Dev* **12**, 2263-2268.

97. Walden H, Podgorski MS, Huang DT, Miller DW, Howard RJ, Minor DL, Jr., Holton JM & Schulman BA (2003) The structure of the APPBP1-UBA3-NEDD8-ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1. *Mol Cell* **12**, 1427-1437, doi: S1097276503004520 [pii].

98. Huang DT, Miller DW, Mathew R, Cassell R, Holton JM, Roussel MF & Schulman BA (2004) A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8. *Nat Struct Mol Biol* **11**, 927-935, doi: 10.1038/nsmb826

nsmb826 [pii].

99. Kurz T, Chou YC, Willems AR, Meyer-Schaller N, Hecht ML, Tyers M, Peter M & Sicheri F (2008) Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation. *Mol Cell* **29**, 23-35, doi: S1097-2765(07)00887-8 [pii]

10.1016/j.molcel.2007.12.012.

100. Yang X, Zhou J, Sun L, Wei Z, Gao J, Gong W, Xu RM, Rao Z & Liu Y (2007) Structural basis for the function of DCN-1 in protein Neddylation. *J Biol Chem* **282**, 24490-24494, doi: C700038200 [pii]

10.1074/jbc.C700038200.

101. Kurz T, Ozlu N, Rudolf F, O'Rourke SM, Luke B, Hofmann K, Hyman AA, Bowerman B & Peter M (2005) The conserved protein DCN-1/Dcn1p is required for cullin neddylation in C. elegans and S. cerevisiae. *Nature* **435**, 1257-1261, doi: nature03662 [pii]

10.1038/nature03662.

102. Xirodimas DP, Saville MK, Bourdon JC, Hay RT & Lane DP (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118**, 83-97, doi: 10.1016/j.cell.2004.06.016

S0092867404005859 [pii].

103. Oved S, Mosesson Y, Zwang Y, Santonico E, Shtiegman K, Marmor MD, Kochupurakkal BS, Katz M, Lavi S, Cesareni G, et al. (2006) Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. *J Biol Chem* **281**, 21640-21651, doi: M513034200 [pii]

10.1074/jbc.M513034200.

104. Broemer M, Tenev T, Rigbolt KT, Hempel S, Blagoev B, Silke J, Ditzel M & Meier P Systematic in vivo RNAi analysis identifies IAPs as NEDD8-E3 ligases. *Mol Cell* **40**, 810-822, doi: S1097-2765(10)00849-X [pii]

10.1016/j.molcel.2010.11.011.

105. Duda DM, Scott DC, Calabrese MF, Zimmerman ES, Zheng N & Schulman BA Structural regulation of cullin-RING ubiquitin ligase complexes. *Curr Opin Struct Biol* **21**, 257-264, doi: S0959-440X(11)00005-4 [pii]

10.1016/j.sbi.2011.01.003.

106. Xirodimas DP, Sundqvist A, Nakamura A, Shen L, Botting C & Hay RT (2008) Ribosomal proteins are targets for the NEDD8 pathway. *EMBO Rep* **9**, 280-286, doi: embor200810 [pii]

10.1038/embor.2008.10.

107. Jones J, Wu K, Yang Y, Guerrero C, Nillegoda N, Pan ZQ & Huang L (2008) A targeted proteomic analysis of the ubiquitin-like modifier nedd8 and associated proteins. *J Proteome Res* **7**, 1274-1287, doi: 10.1021/pr700749v.

108. Ohki Y, Funatsu N, Konishi N & Chiba T (2009) The mechanism of poly-NEDD8 chain formation in vitro. *Biochem Biophys Res Commun* **381**, 443-447, doi: 10.1016/j.bbrc.2009.02.090.

109. Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N & Deshaies RJ (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**, 1382-1385, doi: 10.1126/science.1059780.

110. Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, Kolli N, Gan-Erdene T, Wilkinson KD, Gill G, Lima CD, et al. (2004) Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol Cell Biol* **24**, 84-95.

111. Ferro A, Carvalho AL, Teixeira-Castro A, Almeida C, Tome RJ, Cortes L, Rodrigues AJ, Logarinho E, Sequeiros J, Macedo-Ribeiro S, et al. (2007) NEDD8: a new ataxin-3 interactor. *Biochim Biophys Acta* **1773**, 1619-1627, doi: 10.1016/j.bbamcr.2007.07.012.

112. Artavanis-Tsakonas K, Misaghi S, Comeaux CA, Catic A, Spooner E, Duraisingh MT & Ploegh HL (2006) Identification by functional proteomics of a deubiquitinating/deNeddylating enzyme in Plasmodium falciparum. *Mol Microbiol* **61**, 1187-1195, doi: 10.1111/j.1365-2958.2006.05307.x.

113. Gong L, Kamitani T, Millas S & Yeh ET (2000) Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. *J Biol Chem* **275**, 14212-14216.

114. Ye Y, Akutsu M, Reyes-Turcu F, Enchev RI, Wilkinson KD & Komander D (2011) Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21. *EMBO Rep* **12**, 350-357, doi: 10.1038/embor.2011.17.

115. Kamitani T, Kito K, Fukuda-Kamitani T & Yeh ET (2001) Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1. *J Biol Chem* **276**, 46655-46660, doi: 10.1074/jbc.M108636200

M108636200 [pii].

116. Tanji K, Tanaka T & Kamitani T (2005) Interaction of NUB1 with the proteasome subunit S5a. *Biochem Biophys Res Commun* **337**, 116-120, doi: S0006-291X(05)02013-9 [pii]

10.1016/j.bbrc.2005.09.014.

117. Kito K, Yeh ET & Kamitani T (2001) NUB1, a NEDD8-interacting protein, is induced by interferon and down-regulates the NEDD8 expression. *J Biol Chem* **276**, 20603-20609, doi: 10.1074/jbc.M100920200.

118. Lammer D, Mathias N, Laplaza JM, Jiang W, Liu Y, Callis J, Goebl M & Estelle M (1998) Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. *Genes Dev* **12**, 914-926.

119. Osaka F, Kawasaki H, Aida N, Saeki M, Chiba T, Kawashima S, Tanaka K & Kato S (1998) A new NEDD8-ligating system for cullin-4A. *Genes Dev* **12**, 2263-2268.

120. Handeli S & Weintraub H (1992) The ts41 mutation in Chinese hamster cells leads to successive S phases in the absence of intervening G2, M, and G1. *Cell* **71**, 599-611.

121. Pozo JC, Timpte C, Tan S, Callis J & Estelle M (1998) The ubiquitin-related protein RUB1 and auxin response in Arabidopsis. *Science* **280**, 1760-1763.

122. Jones D & Candido EP (2000) The NED-8 conjugating system in Caenorhabditis elegans is required for embryogenesis and terminal differentiation of the hypodermis. *Dev Biol* **226**, 152-165, doi: 10.1006/dbio.2000.9847.

123. Kurz T, Pintard L, Willis JH, Hamill DR, Gonczy P, Peter M & Bowerman B (2002) Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. *Science* **295**, 1294-1298, doi: 10.1126/science.1067765.

124. Gao MX, Liao EH, Yu B, Wang Y, Zhen M & Derry WB (2008) The SCF FSN-1 ubiquitin ligase controls germline apoptosis through CEP-1/p53 in C. elegans. *Cell Death Differ* **15**, 1054-1062, doi: 10.1038/cdd.2008.30.

125. Petroski MD & Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6**, 9-20, doi: nrm1547 [pii]

10.1038/nrm1547.

126. Skaar JR, Florens L, Tsutsumi T, Arai T, Tron A, Swanson SK, Washburn MP & DeCaprio JA (2007) PARC and CUL7 form atypical cullin RING ligase complexes. *Cancer Res* **67**, 2006-2014, doi: 10.1158/0008-5472.CAN-06-3241.

127. Zimmerman ES, Schulman BA & Zheng N (2010) Structural assembly of cullin-RING ubiquitin ligase complexes. *Curr Opin Struct Biol* **20**, 714-721, doi: 10.1016/j.sbi.2010.08.010.

128. Duda DM, Borg LA, Scott DC, Hunt HW, Hammel M & Schulman BA (2008) Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. *Cell* **134**, 995-1006, doi: 10.1016/j.cell.2008.07.022.

129. Wimuttisuk W & Singer JD (2007) The Cullin3 ubiquitin ligase functions as a Nedd8-bound heterodimer. *Mol Biol Cell* **18**, 899-909, doi: 10.1091/mbc.E06-06-0542.

130. Huang DT, Ayrault O, Hunt HW, Taherbhoy AM, Duda DM, Scott DC, Borg LA, Neale G, Murray PJ, Roussel MF, et al. (2009) E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. *Mol Cell* **33**, 483-495, doi: 10.1016/j.molcel.2009.01.011.

131. Scott DC, Monda JK, Grace CR, Duda DM, Kriwacki RW, Kurz T & Schulman BA (2010) A dual E3 mechanism for Rub1 ligation to Cdc53. *Mol Cell* **39**, 784-796, doi: 10.1016/j.molcel.2010.08.030.

132. Cope GA, Suh GS, Aravind L, Schwarz SE, Zipursky SL, Koonin EV & Deshaies RJ (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608-611, doi: 10.1126/science.1075901.

133. Peng Z, Shen Y, Feng S, Wang X, Chitteti BN, Vierstra RD & Deng XW (2003) Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases in vivo. *Curr Biol* **13**, R504-505.

134. Huang X, Hetfeld BK, Seifert U, Kahne T, Kloetzel PM, Naumann M, Bech-Otschir D & Dubiel W (2005) Consequences of COP9 signalosome and 26S proteasome interaction. *Febs J* **272**, 3909-3917, doi: 10.1111/j.1742-4658.2005.04807.x.

135. Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, Wei N, Sun H, Kobayashi R & Zhang H (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol Cell* **10**, 1519-1526, doi: S1097276502007840 [pii].

136. Bennett EJ, Rush J, Gygi SP & Harper JW (2010) Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* **143**, 951-965, doi: 10.1016/j.cell.2010.11.017.

137. Chuang HW, Zhang W & Gray WM (2004) Arabidopsis ETA2, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF(TIR1) ubiquitin ligase. *Plant Cell* **16**, 1883-1897, doi: 10.1105/tpc.021923.

138. Bosu DR, Feng H, Min K, Kim Y, Wallenfang MR & Kipreos ET (2010) C. elegans CAND-1 regulates cullin neddylation, cell proliferation and morphogenesis in specific tissues. *Dev Biol* **346**, 113-126, doi: 10.1016/j.ydbio.2010.07.020.

139. Cui J, Yao Q, Li S, Ding X, Lu Q, Mao H, Liu L, Zheng N, Chen S & Shao F (2010) Glutamine deamidation and dysfunction of ubiquitin/NEDD8 induced by a bacterial effector family. *Science* **329**, 1215-1218, doi: 10.1126/science.1193844.

140. Abida WM, Nikolaev A, Zhao W, Zhang W & Gu W (2007) FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity. *J Biol Chem* **282**, 1797-1804, doi: 10.1074/jbc.M609001200.

141. Carter S, Bischof O, Dejean A & Vousden KH (2007) C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. *Nat Cell Biol* **9**, 428-435, doi: 10.1038/ncb1562.

142. Liu G & Xirodimas DP NUB1 promotes cytoplasmic localization of p53 through cooperation of the NEDD8 and ubiquitin pathways. *Oncogene*, doi: onc2009494 [pii]

10.1038/onc.2009.494.

143. Dohmesen C, Koeppel M & Dobbelstein M (2008) Specific inhibition of Mdm2-mediated neddylation by Tip60. *Cell Cycle* **7**, 222-231.

144. Watson IR, Blanch A, Lin DC, Ohh M & Irwin MS (2006) Mdm2-mediated NEDD8 modification of TAp73 regulates its transactivation function. *J Biol Chem* **281**, 34096-34103, doi: 10.1074/jbc.M603654200.

145. Petroski MD (2008) The ubiquitin system, disease, and drug discovery. *BMC Biochem* **9 Suppl 1**, S7, doi: 10.1186/1471-2091-9-S1-S7.

146. Gao F, Cheng J, Shi T & Yeh ET (2006) Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFkappaB-dependent transcription. *Nat Cell Biol* **8**, 1171-1177, doi: ncb1483 [pii]

10.1038/ncb1483.

147. Zhang Y, Wolf GW, Bhat K, Jin A, Allio T, Burkhart WA & Xiong Y (2003) Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol Cell Biol* **23**, 8902-8912.

148. Sundqvist A, Liu G, Mirsaliotis A & Xirodimas DP (2009) Regulation of nucleolar signalling to p53 through NEDDylation of L11. *EMBO Rep* **10**, 1132-1139, doi: embor2009178 [pii]

10.1038/embor.2009.178.

149. Choo YS, Vogler G, Wang D, Kalvakuri S, Iliuk A, Tao WA, Bodmer R & Zhang Z (2012) Regulation of parkin and PINK1 by neddylation. *Hum Mol Genet* **21**, 2514-2523, doi: 10.1093/hmg/dds070.

150. Loftus SJ, Liu G, Carr SM, Munro S & La Thangue NB (2012) NEDDylation regulates E2F-1-dependent transcription. *EMBO Rep* **13**, 811-818, doi: 10.1038/embor.2012.113.

151. Aoki I, Higuchi M & Gotoh Y (2012) NEDDylation controls the target specificity of E2F1 and apoptosis induction. *Oncogene*, doi: 10.1038/onc.2012.428.

152. Iyer LM, Burroughs AM & Aravind L (2006) The prokaryotic antecedents of the ubiquitin-signaling system and the early evolution of ubiquitin-like beta-grasp domains. *Genome Biol* **7**, R60, doi: 10.1186/gb-2006-7-7-r60.

153. Welchman RL, Gordon C & Mayer RJ (2005) Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat Rev Mol Cell Biol* **6**, 599-609, doi: nrm1700 [pii]

10.1038/nrm1700.

154. Durfee LA, Lyon N, Seo K & Huibregtse JM (2010) The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15. *Mol Cell* **38**, 722-732, doi: 10.1016/j.molcel.2010.05.002.

155. Hay RT (2005) SUMO: a history of modification. *Mol Cell* **18**, 1-12, doi: 10.1016/j.molcel.2005.03.012.

156. Gareau JR & Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* **11**, 861-871, doi: 10.1038/nrm3011.

157. Geiss-Friedlander R & Melchior F (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-956, doi: 10.1038/nrm2293.

158. Hay RT (2007) SUMO-specific proteases: a twist in the tail. *Trends Cell Biol* **17**, 370-376, doi: 10.1016/j.tcb.2007.08.002.

159. Mahajan R, Delphin C, Guan T, Gerace L & Melchior F (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97-107.

160. Matunis MJ, Coutavas E & Blobel G (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* **135**, 1457-1470.

161. Saitoh H & Hinchey J (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-6258.

162. Kerscher O (2007) SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep* **8**, 550-555, doi: 10.1038/sj.embor.7400980.

163. Geoffroy MC & Hay RT (2009) An additional role for SUMO in ubiquitin-mediated proteolysis. *Nat Rev Mol Cell Biol* **10**, 564-568, doi: 10.1038/nrm2707.

164. Girdwood DW, Tatham MH & Hay RT (2004) SUMO and transcriptional regulation. *Semin Cell Dev Biol* **15**, 201-210.

165. Lieberman AP (2004) SUMO, a ubiquitin-like modifier implicated in neurodegeneration. *Exp Neurol* **185**, 204-207.

166. Baek SH (2006) A novel link between SUMO modification and cancer metastasis. *Cell Cycle* **5**, 1492-1495.

167. Furukawa K, Mizushima N, Noda T & Ohsumi Y (2000) A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *J Biol Chem* **275**, 7462-7465.

168. Goehring AS, Rivers DM & Sprague GF, Jr. (2003) Urmylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell* **14**, 4329-4341, doi: 10.1091/mbc.E03-02-0079.

169. Van der Veen AG, Schorpp K, Schlieker C, Buti L, Damon JR, Spooner E, Ploegh HL & Jentsch S (2011) Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *Proc Natl Acad Sci U S A* **108**, 1763-1770, doi: 10.1073/pnas.1014402108.

170. Wang F, Liu M, Qiu R & Ji C (2011) The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. *Protein Cell* **2**, 612-619, doi: 10.1007/s13238-011-1074-6.

171. Fan W, Cai W, Parimoo S, Schwarz DC, Lennon GG & Weissman SM (1996) Identification of seven new human MHC class I region genes around the HLA-F locus. *Immunogenetics* **44**, 97-103.

172. Hipp MS, Raasi S, Groettrup M & Schmidtke G (2004) NEDD8 ultimate buster-1L interacts with the ubiquitin-like protein FAT10 and accelerates its degradation. *J Biol Chem* **279**, 16503-16510, doi: 10.1074/jbc.M310114200.

173. Aichem A, Pelzer C, Lukasiak S, Kalveram B, Sheppard PW, Rani N, Schmidtke G & Groettrup M (2010) USE1 is a bispecific conjugating enzyme for ubiquitin and FAT10, which FAT10ylates itself in cis. *Nat Commun* **1**, 13, doi: 10.1038/ncomms1012.

174. Li T, Santockyte R, Yu S, Shen RF, Tekle E, Lee CG, Yang DC & Chock PB (2011) FAT10 modifies p53 and upregulates its transcriptional activity. *Arch Biochem Biophys* **509**, 164-169, doi: 10.1016/j.abb.2011.02.017.

175. Rani N, Aichem A, Schmidtke G, Kreft SG & Groettrup M (2012) FAT10 and NUB1L bind to the VWA domain of Rpn10 and Rpn1 to enable proteasome-mediated proteolysis. *Nat Commun* **3**, 749, doi: 10.1038/ncomms1752.

176. Aichem A, Kalveram B, Spinnenhirn V, Kluge K, Catone N, Johansen T & Groettrup M (2012) The proteomic analysis of endogenous FAT10 substrates identifies p62/SQSTM1 as a substrate of FAT10ylation. *J Cell Sci*, doi: 10.1242/jcs.107789.

177. Liu YC, Pan J, Zhang C, Fan W, Collinge M, Bender JR & Weissman SM (1999) A MHC-encoded ubiquitin-like protein (FAT10) binds noncovalently to the spindle assembly checkpoint protein MAD2. *Proc Natl Acad Sci U S A* **96**, 4313-4318.

178. Raasi S, Schmidtke G, de Giuli R & Groettrup M (1999) A ubiquitin-like protein which is synergistically inducible by interferon-gamma and tumor necrosis factor-alpha. *Eur J Immunol* **29**, 4030-4036, doi: 10.1002/(SICI)1521-4141(199912)29:12<4030::AID-IMMU4030>3.0.CO;2-Y.

179. Bates EE, Ravel O, Dieu MC, Ho S, Guret C, Bridon JM, Ait-Yahia S, Briere F, Caux C, Banchereau J, et al. (1997) Identification and analysis of a novel member of the ubiquitin family expressed in dendritic cells and mature B cells. *Eur J Immunol* **27**, 2471-2477, doi: 10.1002/eji.1830271002.

180. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M & Ohsumi Y (1998) A protein conjugation system essential for autophagy. *Nature* **395**, 395-398, doi: 10.1038/26506.

181. Mizushima N, Sugita H, Yoshimori T & Ohsumi Y (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J Biol Chem* **273**, 33889-33892.

182. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, Ohsumi M, Takao T, Noda T & Ohsumi Y (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol* **151**, 263-276.

183. McNally T, Huang Q, Janis RS, Liu Z, Olejniczak ET & Reilly RM (2003) Structural analysis of UBL5, a novel ubiquitin-like modifier. *Protein Sci* **12**, 1562-1566, doi: 10.1110/ps.0382803.

184. Ramelot TA, Cort JR, Yee AA, Semesi A, Edwards AM, Arrowsmith CH & Kennedy MA (2003) Solution structure of the yeast ubiquitin-like modifier protein Hub1. *J Struct Funct Genomics* **4**, 25-30.

185. Mishra SK, Ammon T, Popowicz GM, Krajewski M, Nagel RJ, Ares M, Jr., Holak TA & Jentsch S (2011) Role of the ubiquitin-like protein Hub1 in splice-site usage and alternative splicing. *Nature* **474**, 173-178, doi: 10.1038/nature10143.

186. Benedetti C, Haynes CM, Yang Y, Harding HP & Ron D (2006) Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. *Genetics* **174**, 229-239, doi: 10.1534/genetics.106.061580.

187. Dittmar GA, Wilkinson CR, Jedrzejewski PT & Finley D (2002) Role of a ubiquitin-like modification in polarized morphogenesis. *Science* **295**, 2442-2446, doi: 10.1126/science.1069989.

188. Pearce MJ, Mintseris J, Ferreyra J, Gygi SP & Darwin KH (2008) Ubiquitin-like protein involved in the proteasome pathway of Mycobacterium tuberculosis. *Science* **322**, 1104-1107, doi: 10.1126/science.1163885.

189. Burns KE, Cerda-Maira FA, Wang T, Li H, Bishai WR & Darwin KH (2010) "Depupylation" of prokaryotic ubiquitin-like protein from mycobacterial proteasome substrates. *Mol Cell* **39**, 821-827, doi: 10.1016/j.molcel.2010.07.019.

190. Imkamp F, Striebel F, Sutter M, Ozcelik D, Zimmermann N, Sander P & Weber-Ban E (2010) Dop functions as a depupylase in the prokaryotic ubiquitin-like modification pathway. *EMBO Rep* **11**, 791-797, doi: 10.1038/embor.2010.119.

191. Bedford L, Lowe J, Dick LR, Mayer RJ & Brownell JE (2011) Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nat Rev Drug Discov* **10**, 29-46, doi: 10.1038/nrd3321.

192. Yang XJ (2005) Multisite protein modification and intramolecular signaling. *Oncogene* **24**, 1653-1662, doi: 10.1038/sj.onc.1208173.

193. Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ & Kim KW (2004) Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem Biophys Res Commun* **324**, 394-400, doi: 10.1016/j.bbrc.2004.09.068.

194. Papouli E, Chen S, Davies AA, Huttner D, Krejci L, Sung P & Ulrich HD (2005) Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Mol Cell* **19**, 123-133, doi: 10.1016/j.molcel.2005.06.001.

195. Richter K, Haslbeck M & Buchner J The heat shock response: life on the verge of death. *Mol Cell* **40**, 253-266, doi: S1097-2765(10)00782-3 [pii]

10.1016/j.molcel.2010.10.006.

196. Watt R & Piper PW (1997) UBI4, the polyubiquitin gene of Saccharomyces cerevisiae, is a heat shock gene that is also subject to catabolite derepression control. *Mol Gen Genet* **253**, 439-447.

197. Meulmeester E & Melchior F (2008) Cell biology: SUMO. *Nature* **452**, 709-711, doi: 10.1038/452709a.

198. Golebiowski F, Matic I, Tatham MH, Cole C, Yin Y, Nakamura A, Cox J, Barton GJ, Mann M & Hay RT (2009) System-wide changes to SUMO modifications in response to heat shock. *Sci Signal* **2**, ra24, doi: 2/72/ra24 [pii]

10.1126/scisignal.2000282.

199. Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77**, 755-776, doi: 10.1146/annurev.biochem.77.061606.161055.

200. Bossis G & Melchior F (2006) Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell* **21**, 349-357, doi: S1097-2765(05)01903-9 [pii]

10.1016/j.molcel.2005.12.019.

201. Doris KS, Rumsby EL & Morgan BA (2012) Oxidative stress responses involves oxidation of a conserved ubiquitin pathway enzyme. *Mol Cell Biol*, doi: 10.1128/MCB.00559-12.

202. Hanna J, Leggett DS & Finley D (2003) Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. *Mol Cell Biol* **23**, 9251-9261.

203. Chernova TA, Allen KD, Wesoloski LM, Shanks JR, Chernoff YO & Wilkinson KD (2003) Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. *J Biol Chem* **278**, 52102-52115, doi: 10.1074/jbc.M310283200.

204. Frankland-Searby S & Bhaumik SR (2012) The 26S proteasome complex: an attractive target for cancer therapy. *Biochim Biophys Acta* **1825**, 64-76, doi: 10.1016/j.bbcan.2011.10.003.

205. Walsh RC, Everly JJ, Brailey P, Rike AH, Arend LJ, Mogilishetty G, Govil A, Roy-Chaudhury P, Alloway RR & Woodle ES (2010) Proteasome inhibitor-based primary therapy for antibody-mediated renal allograft rejection. *Transplantation* **89**, 277-284, doi: 10.1097/TP.0b013e3181c6ff8d.

206. Guardavaccaro D & Pagano M (2004) Oncogenic aberrations of cullin-dependent ubiquitin ligases. *Oncogene* **23**, 2037-2049, doi: 10.1038/sj.onc.1207413.

207. Chairatvit K & Ngamkitidechakul C (2007) Control of cell proliferation via elevated NEDD8 conjugation in oral squamous cell carcinoma. *Mol Cell Biochem* **306**, 163-169, doi: 10.1007/s11010-007-9566-7.

208. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, et al. (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* **458**, 732-736, doi: nature07884 [pii]

10.1038/nature07884.

209. Brownell JE, Sintchak MD, Gavin JM, Liao H, Bruzzese FJ, Bump NJ, Soucy TA, Milhollen MA, Yang X, Burkhardt AL, et al. Substrate-assisted inhibition of ubiquitin-like protein-activating enzymes: the NEDD8 E1 inhibitor MLN4924 forms a NEDD8-AMP mimetic in situ. *Mol Cell* **37**, 102-111, doi: S1097-2765(09)00955-1 [pii]

10.1016/j.molcel.2009.12.024.

210. Swords RT, Kelly KR, Smith PG, Garnsey JJ, Mahalingam D, Medina E, Oberheu K, Padmanabhan S, O'Dwyer M, Nawrocki ST, et al. (2010) Inhibition of

NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. *Blood* **115**, 3796-3800, doi: 10.1182/blood-2009-11-254862.

211. Cohn MA, Kowal P, Yang K, Haas W, Huang TT, Gygi SP & D'Andrea AD (2007) A UAF1-containing multisubunit protein complex regulates the Fanconi anemia pathway. *Mol Cell* **28**, 786-797, doi: 10.1016/j.molcel.2007.09.031.

212. Masuya D, Huang C, Liu D, Nakashima T, Yokomise H, Ueno M, Nakashima N & Sumitomo S (2006) The HAUSP gene plays an important role in non-small cell lung carcinogenesis through p53-dependent pathways. *J Pathol* **208**, 724-732, doi: 10.1002/path.1931.

213. Liu Y, Fallon L, Lashuel HA, Liu Z & Lansbury PT, Jr. (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* **111**, 209-218.

214. Edelmann MJ & Kessler BM (2008) Ubiquitin and ubiquitin-like specific proteases targeted by infectious pathogens: Emerging patterns and molecular principles. *Biochim Biophys Acta* **1782**, 809-816, doi: 10.1016/j.bbadis.2008.08.010.

215. Chen Y, Liu W, McPhie DL, Hassinger L & Neve RL (2003) APP-BP1 mediates APP-induced apoptosis and DNA synthesis and is increased in Alzheimer's disease brain. *J Cell Biol* **163**, 27-33, doi: 10.1083/jcb.200304003.

216. Chen Y, Liu W, Naumovski L & Neve RL (2003) ASPP2 inhibits APP-BP1-mediated NEDD8 conjugation to cullin-1 and decreases APP-BP1-induced cell proliferation and neuronal apoptosis. *J Neurochem* **85**, 801-809.

217. Davies JE, Sarkar S & Rubinsztein DC (2007) The ubiquitin proteasome system in Huntington's disease and the spinocerebellar ataxias. *BMC Biochem* **8 Suppl 1**, S2, doi: 10.1186/1471-2091-8-S1-S2.

218. Upadhya SC & Hegde AN (2007) Role of the ubiquitin proteasome system in Alzheimer's disease. *BMC Biochem* **8 Suppl 1**, S12, doi: 10.1186/1471-2091-8-S1-S12.

219. Herrmann J, Lerman LO & Lerman A (2007) Ubiquitin and ubiquitin-like proteins in protein regulation. *Circ Res* **100**, 1276-1291, doi: 10.1161/01.RES.0000264500.11888.f0.

220. Tsukamoto O, Minamino T, Okada K, Shintani Y, Takashima S, Kato H, Liao Y, Okazaki H, Asai M, Hirata A, et al. (2006) Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice. *Biochem Biophys Res Commun* **340**, 1125-1133, doi: 10.1016/j.bbrc.2005.12.120.

221. Zhang C, Xu Z, He XR, Michael LH & Patterson C (2005) CHIP, a cochaperone/ubiquitin ligase that regulates protein quality control, is required for maximal cardioprotection after myocardial infarction in mice. *Am J Physiol Heart Circ Physiol* **288**, H2836-2842, doi: 10.1152/ajpheart.01122.2004.

222. Huen MS & Chen J (2008) The DNA damage response pathways: at the crossroad of protein modifications. *Cell Res* **18**, 8-16, doi: 10.1038/cr.2007.109.

223. Povlsen LK, Beli P, Wagner SA, Poulsen SL, Sylvestersen KB, Poulsen JW, Nielsen ML, Bekker-Jensen S, Mailand N & Choudhary C (2012) Systems-wide analysis of ubiquitylation dynamics reveals a key role for PAF15 ubiquitylation in DNA-damage bypass. *Nat Cell Biol* **14**, 1089-1098, doi: 10.1038/ncb2579.

224. Sansam CL, Shepard JL, Lai K, Ianari A, Danielian PS, Amsterdam A, Hopkins N & Lees JA (2006) DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint. *Genes Dev* **20**, 3117-3129, doi: 10.1101/gad.1482106.

225. Jin J, Arias EE, Chen J, Harper JW & Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* **23**, 709-721, doi: 10.1016/j.molcel.2006.08.010.

226. Milhollen MA, Narayanan U, Soucy TA, Veiby PO, Smith PG & Amidon B (2011) Inhibition of NEDD8-activating enzyme induces rereplication and apoptosis in human tumor cells consistent with deregulating CDT1 turnover. *Cancer Res* **71**, 3042-3051, doi: 10.1158/0008-5472.CAN-10-2122.

227. Alpi AF & Patel KJ (2009) Monoubiquitylation in the Fanconi anemia DNA damage response pathway. *DNA Repair (Amst)* **8**, 430-435, doi: 10.1016/j.dnarep.2009.01.019.

228. MacKay C, Declais AC, Lundin C, Agostinho A, Deans AJ, MacArtney TJ, Hofmann K, Gartner A, West SC, Helleday T, et al. (2010) Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell* **142**, 65-76, doi: 10.1016/j.cell.2010.06.021.

229. Mattiroli F, Vissers JH, van Dijk WJ, Ikpa P, Citterio E, Vermeulen W, Marteijn JA & Sixma TK (2012) RNF168 Ubiquitinates K13-15 on H2A/H2AX to Drive DNA Damage Signaling. *Cell* **150**, 1182-1195, doi: 10.1016/j.cell.2012.08.005.

230. Galanty Y, Belotserkovskaya R, Coates J, Polo S, Miller KM & Jackson SP (2009) Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* **462**, 935-939, doi: 10.1038/nature08657.

231. Yin Y, Seifert A, Chua JS, Maure JF, Golebiowski F & Hay RT (2012) SUMO-targeted ubiquitin E3 ligase RNF4 is required for the response of human cells to DNA damage. *Genes Dev* **26**, 1196-1208, doi: 10.1101/gad.189274.112.

232. Wyllie AH, Kerr JF & Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* **68**, 251-306.

233. Horvitz HR (1999) Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Res* **59**, 1701s-1706s.

234. Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516, doi: 10.1080/01926230701320337.

235. Walczak H & Krammer PH (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental cell research* **256**, 58-66, doi: 10.1006/excr.2000.4840.

236. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, et al. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* **85**, 817-827.

237. Chinnaiyan AM (1999) The apoptosome: heart and soul of the cell death machine. *Neoplasia* **1**, 5-15.

238. Shi Y (2004) Caspase activation, inhibition, and reactivation: a mechanistic view. *Protein science : a publication of the Protein Society* **13**, 1979-1987, doi: 10.1110/ps.04789804.

239. Salvesen GS & Duckett CS (2002) IAP proteins: blocking the road to death's door. *Nature reviews Molecular cell biology* **3**, 401-410, doi: 10.1038/nrm830.

240. Shi Y (2002) Mechanisms of caspase activation and inhibition during apoptosis. *Molecular cell* **9**, 459-470.

241. Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, MacFarlane M, Cain K, et al. (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell* **43**, 432-448, doi: 10.1016/j.molcel.2011.06.006.

242. Lee JC & Peter ME (2003) Regulation of apoptosis by ubiquitination. *Immunol Rev* **193**, 39-47.

243. Broemer M & Meier P (2009) Ubiquitin-mediated regulation of apoptosis. *Trends Cell Biol* **19**, 130-140, doi: 10.1016/j.tcb.2009.01.004.

244. Lohrum MA, Woods DB, Ludwig RL, Balint E & Vousden KH (2001) C-terminal ubiquitination of p53 contributes to nuclear export. *Mol Cell Biol* **21**, 8521-8532, doi: 10.1128/MCB.21.24.8521-8532.2001.

245. Vousden KH & Lane DP (2007) p53 in health and disease. *Nat Rev Mol Cell Biol* **8**, 275-283, doi: 10.1038/nrm2147.

246. Zhao L, Yue P, Lonial S, Khuri FR & Sun SY (2011) The NEDD8-activating enzyme inhibitor, MLN4924, cooperates with TRAIL to augment apoptosis through facilitating c-FLIP degradation in head and neck cancer cells. *Mol Cancer Ther* **10**, 2415-2425, doi: 10.1158/1535-7163.MCT-11-0401.

247. Luo Z, Yu G, Lee HW, Li L, Wang L, Yang D, Pan Y, Ding C, Qian J, Wu L, et al. (2012) The Nedd8-activating enzyme inhibitor MLN4924 induces autophagy and apoptosis to suppress liver cancer cell growth. *Cancer Res* **72**, 3360-3371, doi: 10.1158/0008-5472.CAN-12-0388.

248. Rappsilber J, Mann M & Ishihama Y (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**, 1896-1906, doi: nprot.2007.261 [pii]

10.1038/nprot.2007.261.

249. Cox J & Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**, 1367-1372, doi: nbt.1511 [pii]

10.1038/nbt.1511.

250. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV & Mann M Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* **10**, 1794-1805, doi: 10.1021/pr101065j.

251. Forsburg SL & Rhind N (2006) Basic methods for fission yeast. *Yeast* **23**, 173-183, doi: 10.1002/yea.1347.

252. Eisen MB, Spellman PT, Brown PO & Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* **95**, 14863-14868.

253. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D & Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11**, 4241-4257.

254. Parag HA, Raboy B & Kulka RG (1987) Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system. *Embo J* **6**, 55-61.

255. Zhang X, Zhou J, Fernandes AF, Sparrow JR, Pereira P, Taylor A & Shang F (2008) The proteasome: a target of oxidative damage in cultured human retina pigment epithelial cells. *Invest Ophthalmol Vis Sci* **49**, 3622-3630, doi: iovs.07-1559 [pii]

10.1167/iovs.07-1559.

256. Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY & Vierstra RD (2003) The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis. Accumulation of SUMO1 and -2 conjugates is increased by stress. *J Biol Chem* **278**, 6862-6872, doi: 10.1074/jbc.M209694200.

257. Saitoh H & Hinchey J (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-6258.

258. Schimmel J, Larsen KM, Matic I, van Hagen M, Cox J, Mann M, Andersen JS & Vertegaal AC (2008) The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. *Mol Cell Proteomics* **7**, 2107-2122, doi: M800025-MCP200 [pii]

10.1074/mcp.M800025-MCP200.

259. Tatham MH, Matic I, Mann M & Hay RT Comparative proteomic analysis identifies a role for SUMO in protein quality control. *Sci Signal* **4**, rs4, doi: 4/178/rs4 [pii]

10.1126/scisignal.2001484.

260. Hjerpe R, Thomas Y, Chen J, Zemla A, Curran S, Shpiro N, Dick LR & Kurz T (2012) Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. *Biochem J* **441**, 927-936, doi: 10.1042/BJ20111671.

261. Whitby FG, Xia G, Pickart CM & Hill CP (1998) Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes. *J Biol Chem* **273**, 34983-34991.

262. Lee DH & Goldberg AL (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* **8**, 397-403, doi: S0962-8924(98)01346-4 [pii].

263. Finley D & Chau V (1991) Ubiquitination. *Annu Rev Cell Biol* **7**, 25-69, doi: 10.1146/annurev.cb.07.110191.000325.

264. Huang J, Yan J, Zhang J, Zhu S, Wang Y, Shi T, Zhu C, Chen C, Liu X, Cheng J, et al. (2012) SUMO1 modification of PTEN regulates tumorigenesis by controlling its association with the plasma membrane. *Nat Commun* **3**, 911, doi: 10.1038/ncomms1919.

265. Lee YJ, Johnson KR & Hallenbeck JM (2012) Global Protein Conjugation by Ubiquitin-Like-Modifiers during Ischemic Stress Is Regulated by MicroRNAs and Confers Robust Tolerance to Ischemia. *PLoS One* **7**, e47787, doi: 10.1371/journal.pone.0047787.

266. Mimnaugh EG, Chen HY, Davie JR, Celis JE & Neckers L (1997) Rapid deubiquitination of nucleosomal histones in human tumor cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and the cellular stress response. *Biochemistry* **36**, 14418-14429, doi: 10.1021/bi970998j

bi970998j [pii].

267. Shang F & Taylor A Ubiquitin-proteasome pathway and cellular responses to oxidative stress. *Free Radic Biol Med*, doi: S0891-5849(11)00204-8 [pii]

10.1016/j.freeradbiomed.2011.03.031.

268. Corton JM, Gillespie JG & Hardie DG (1994) Role of the AMP-activated protein kinase in the cellular stress response. *Curr Biol* **4**, 315-324, doi: S0960-9822(00)00070-1 [pii].

269. Wang X, Yen J, Kaiser P & Huang L Regulation of the 26S proteasome complex during oxidative stress. *Sci Signal* **3**, ra88, doi: 3/151/ra88 [pii]

10.1126/scisignal.2001232.

270. Cook JC & Chock PB (1995) Phosphorylation of ubiquitin-activating enzyme in cultured cells. *Proc Natl Acad Sci U S A* **92**, 3454-3457.

271. Nagai Y, Kaneda S, Nomura K, Yasuda H, Seno T & Yamao F (1995) Ubiquitin-activating enzyme, E1, is phosphorylated in mammalian cells by the protein kinase Cdc2. *J Cell Sci* **108 (Pt 6)**, 2145-2152.

272. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV & Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325**, 834-840, doi: 1175371 [pii]

10.1126/science.1175371.

273. Kitahara R, Yamaguchi Y, Sakata E, Kasuya T, Tanaka K, Kato K, Yokoyama S & Akasaka K (2006) Evolutionally conserved intermediates between ubiquitin and NEDD8. *J Mol Biol* **363**, 395-404, doi: S0022-2836(06)00971-5 [pii]

10.1016/j.jmb.2006.07.074.

274. Witze ES, Old WM, Resing KA & Ahn NG (2007) Mapping protein post-translational modifications with mass spectrometry. *Nat Methods* **4**, 798-806, doi: 10.1038/nmeth1100.

275. Vertegaal AC (2011) Uncovering ubiquitin and ubiquitin-like signaling networks. *Chem Rev* **111**, 7923-7940, doi: 10.1021/cr200187e.

276. Golebiowski F, Tatham MH, Nakamura A & Hay RT (2010) High-stringency tandem affinity purification of proteins conjugated to ubiquitin-like moieties. *Nat Protoc* **5**, 873-882, doi: 10.1038/nprot.2010.40.

277. Aillet F, Lopitz-Otsoa F, Hjerpe R, Torres-Ramos M, Lang V & Rodriguez MS (2012) Isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *Methods Mol Biol* **832**, 173-183, doi: 10.1007/978-1-61779-474-2_12.

278. Hjerpe R, Aillet F, Lopitz-Otsoa F, Lang V, England P & Rodriguez MS (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep* **10**, 1250-1258, doi: 10.1038/embor.2009.192.

279. Bruderer R, Tatham MH, Plechanovova A, Matic I, Garg AK & Hay RT (2011) Purification and identification of endogenous polySUMO conjugates. *EMBO Rep* **12**, 142-148, doi: 10.1038/embor.2010.206.

280. Jeram SM, Srikumar T, Pedrioli PG & Raught B (2009) Using mass spectrometry to identify ubiquitin and ubiquitin-like protein conjugation sites. *Proteomics* **9**, 922-934, doi: 10.1002/pmic.200800666.

281. Xu G, Paige JS & Jaffrey SR (2010) Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat Biotechnol* **28**, 868-873, doi: 10.1038/nbt.1654.

282. Udeshi ND, Mani DR, Eisenhaure T, Mertins P, Jaffe JD, Clauser KR, Hacohen N & Carr SA (2012) Methods for quantification of in vivo changes in protein ubiquitination following proteasome and deubiquitinase inhibition. *Mol Cell Proteomics* **11**, 148-159, doi: 10.1074/mcp.M111.016857.

283. Lee KA, Hammerle LP, Andrews PS, Stokes MP, Mustelin T, Silva JC, Black RA & Doedens JR (2011) Ubiquitin ligase substrate identification through quantitative

proteomics at both the protein and peptide levels. *J Biol Chem* **286**, 41530-41538, doi: 10.1074/jbc.M111.248856.

284. Na CH, Jones DR, Yang Y, Wang X, Xu Y & Peng J (2012) Synaptic Protein Ubiquitination in Rat Brain Revealed by Antibody-based Ubiquitome Analysis. *J Proteome Res* **11**, 4722-4732, doi: 10.1021/pr300536k.

285. Gerber SA, Rush J, Stemman O, Kirschner MW & Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci U S A* **100**, 6940-6945, doi: 10.1073/pnas.0832254100.

286. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A & Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* **1**, 376-386.

287. Mann M (2006) Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol* **7**, 952-958, doi: nrm2067 [pii]

10.1038/nrm2067.

288. Ong SE & Mann M (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protoc* **1**, 2650-2660, doi: nprot.2006.427 [pii]

10.1038/nprot.2006.427.

289. Xirodimas DP (2008) Novel substrates and functions for the ubiquitin-like molecule NEDD8. *Biochem Soc Trans* **36**, 802-806, doi: BST0360802 [pii]

10.1042/BST0360802.

290. Leidecker O & Xirodimas DP (2012) Isolation of NEDDylated proteins in human cells. *Methods Mol Biol* **832**, 133-140, doi: 10.1007/978-1-61779-474-2_8.

291. Denis NJ, Vasilescu J, Lambert JP, Smith JC & Figeys D (2007) Tryptic digestion of ubiquitin standards reveals an improved strategy for identifying ubiquitinated proteins by mass spectrometry. *Proteomics* **7**, 868-874, doi: 10.1002/pmic.200600410.

292. Jeram SM, Srikumar T, Zhang XD, Anne Eisenhauer H, Rogers R, Pedrioli PG, Matunis M & Raught B An improved SUMmOn-based methodology for the identification of ubiquitin and ubiquitin-like protein conjugation sites identifies novel ubiquitin-like protein chain linkages. *Proteomics* **10**, 254-265, doi: 10.1002/pmic.200900648.

293. Matic I & Hay RT (2012) Detection and quantitation of SUMO chains by mass spectrometry. *Methods Mol Biol* **832**, 239-247, doi: 10.1007/978-1-61779-474-2_17.

294. Matic I, van Hagen M, Schimmel J, Macek B, Ogg SC, Tatham MH, Hay RT, Lamond AI, Mann M & Vertegaal AC (2008) In vivo identification of human small ubiquitin-like modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. *Mol Cell Proteomics* **7**, 132-144, doi: M700173-MCP200 [pii]

10.1074/mcp.M700173-MCP200.

295. Verma R, Chen S, Feldman R, Schieltz D, Yates J, Dohmen J & Deshaies RJ (2000) Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol Biol Cell* **11**, 3425-3439.

296. Jeram SM, Srikumar T, Zhang XD, Anne Eisenhauer H, Rogers R, Pedrioli PG, Matunis M & Raught B (2010) An improved SUMmOn-based methodology for the identification of ubiquitin and ubiquitin-like protein conjugation sites identifies novel ubiquitin-like protein chain linkages. *Proteomics* **10**, 254-265, doi: 10.1002/pmic.200900648.

297. Komander D & Rape M (2012) The ubiquitin code. *Annu Rev Biochem* **81**, 203-229, doi: 10.1146/annurev-biochem-060310-170328.

298. Xia ZP, Sun L, Chen X, Pineda G, Jiang X, Adhikari A, Zeng W & Chen ZJ (2009) Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature* **461**, 114-119, doi: 10.1038/nature08247.

299. Iwai K & Tokunaga F (2009) Linear polyubiquitination: a new regulator of NF-kappaB activation. *EMBO Rep* **10**, 706-713, doi: embor2009144 [pii]

10.1038/embor.2009.144.

300. Schmidt MH & Dikic I (2006) Ubiquitin and NEDD8: brothers in arms. *Sci STKE* **2006**, pe50, doi: stke.3622006pe50 [pii]

10.1126/stke.3622006pe50.

301. Gong L, Kamitani T, Millas S & Yeh ET (2000) Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. *J Biol Chem* **275**, 14212-14216, doi: 275/19/14212 [pii].

302. Reverter D, Wu K, Erdene TG, Pan ZQ, Wilkinson KD & Lima CD (2005) Structure of a complex between Nedd8 and the Ulp/Senp protease family member Den1. *J Mol Biol* **345**, 141-151, doi: S0022-2836(04)01314-2 [pii]

10.1016/j.jmb.2004.10.022.

303. Reverter D, Wu K, Erdene TG, Pan ZQ, Wilkinson KD & Lima CD (2005) Structure of a complex between Nedd8 and the Ulp/Senp protease family member Den1. *J Mol Biol* **345**, 141-151, doi: 10.1016/j.jmb.2004.10.022.

304. Chan Y, Yoon J, Wu JT, Kim HJ, Pan KT, Yim J & Chien CT (2008) DEN1 deneddylates non-cullin proteins in vivo. *J Cell Sci* **121**, 3218-3223, doi: jcs.030445 [pii]

10.1242/jcs.030445.

305. Sulston JE, Schierenberg E, White JG & Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol* **100**, 64-119.

306. Gartner A, Milstein S, Ahmed S, Hodgkin J & Hengartner MO (2000) A conserved checkpoint pathway mediates DNA damage--induced apoptosis and cell cycle arrest in C. elegans. *Mol Cell* **5**, 435-443.

307. Schumacher B, Hofmann K, Boulton S & Gartner A (2001) The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. *Curr Biol* **11**, 1722-1727.

308. Schumacher B, Schertel C, Wittenburg N, Tuck S, Mitani S, Gartner A, Conradt B & Shaham S (2005) C. elegans ced-13 can promote apoptosis and is induced in response to DNA damage. *Cell Death Differ* **12**, 153-161, doi: 10.1038/sj.cdd.4401539.

309. Hengartner MO & Horvitz HR (1994) Activation of C. elegans cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature* **369**, 318-320, doi: 10.1038/369318a0.

310. Hengartner MO & Horvitz HR (1994) C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76**, 665-676.

311. Hengartner MO, Ellis RE & Horvitz HR (1992) Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature* **356**, 494-499, doi: 10.1038/356494a0.

312. Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X & Akey CW (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* **9**, 423-432.

313. Pourkarimi E, Greiss S & Gartner A (2012) Evidence that CED-9/Bcl2 and CED-4/Apaf-1 localization is not consistent with the current model for C. elegans apoptosis induction. *Cell Death Differ* **19**, 406-415, doi: 10.1038/cdd.2011.104.

314. Watson IR, Li BK, Roche O, Blanch A, Ohh M & Irwin MS Chemotherapy induces NEDP1-mediated destabilization of MDM2. *Oncogene* **29**, 297-304, doi: onc2009314 [pii]

10.1038/onc.2009.314.

315. Bossy-Wetzel E & Green DR (2000) Detection of apoptosis by annexin V labeling. *Methods in enzymology* **322**, 15-18.

316. Larance M, Bailly AP, Pourkarimi E, Hay RT, Buchanan G, Coulthurst S, Xirodimas DP, Gartner A & Lamond AI (2011) Stable-isotope labeling with amino acids in nematodes. *Nat Methods* **8**, 849-851, doi: 10.1038/nmeth.1679.

317. Blattner C, Sparks A & Lane D (1999) Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. *Mol Cell Biol* **19**, 3704-3713.

318. Biswas AK & Johnson DG (2012) Transcriptional and nontranscriptional functions of E2F1 in response to DNA damage. *Cancer Res* **72**, 13-17, doi: 10.1158/0008-5472.CAN-11-2196.

319. Schertel C & Conradt B (2007) C. elegans orthologs of components of the RB tumor suppressor complex have distinct pro-apoptotic functions. *Development* **134**, 3691-3701, doi: 10.1242/dev.004606.

320. Dahlmann B (2007) Role of proteasomes in disease. *BMC Biochem* **8 Suppl 1**, S3, doi: 1471-2091-8-S1-S3 [pii]

10.1186/1471-2091-8-S1-S3.

321. Dil Kuazi A, Kito K, Abe Y, Shin RW, Kamitani T & Ueda N (2003) NEDD8 protein is involved in ubiquitinated inclusion bodies. *J Pathol* **199**, 259-266, doi: 10.1002/path.1283.

322. Mori F, Nishie M, Piao YS, Kito K, Kamitani T, Takahashi H & Wakabayashi K (2005) Accumulation of NEDD8 in neuronal and glial inclusions of neurodegenerative disorders. *Neuropathol Appl Neurobiol* **31**, 53-61, doi: NAN603 [pii]

10.1111/j.1365-2990.2004.00603.x.

323. Hjerpe R, Thomas Y & Kurz T (2012) NEDD8 overexpression results in neddylation of ubiquitin substrates by the ubiquitin pathway. *J Mol Biol* **421**, 27-29, doi: 10.1016/j.jmb.2012.05.013.

324. Chen Y, Neve RL & Liu H (2012) Neddylation Dysfunction in Alzheimer's Disease. *J Cell Mol Med*, doi: 10.1111/j.1582-4934.2012.01604.x.

325. Pan ZQ, Kentsis A, Dias DC, Yamoah K & Wu K (2004) Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* **23**, 1985-1997, doi: 10.1038/sj.onc.1207414.